ELECTROPHYSIOLOGICAL STUDY OF FRESHLY ISOLATED ARTICULAR CHONDROCYTES vs. CRYOPRESERVED CHONDROCYTES

A Dissertation submitted in partial fulfilment of the requirement for the

Degree of Doctor of Medicine in Physiology (Branch – V) Of the

Tamil Nadu Dr. M.G.R Medical University, Chennai -600 032

Department of Physiology

Christian Medical College, Vellore,

Tamil Nadu

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This is to certify that the thesis entitled "**Electrophysiological study of freshly isolated articular chondrocytes vs. cryopreserved chondrocytes**" is a bonafide, original work carried out by Dr. Upasana Kachroo, in partial fulfillment of the rules and regulations for the M.D – Branch V Physiology examination of the Tamilnadu Dr. M.G.R. Medical University, Chennai to be held in April- 2015.

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DECLARATION

I hereby declare that the investigations that form the subject matter for the thesis entitled "**Electrophysiological study of freshly isolated articular chondrocytes vs. cryopreserved chondrocytes**" were carried out by me during my term as a post graduate student in the Department of Physiology, Christian Medical College, Vellore. This thesis has not been submitted in part or full to any other university.

Upasana 27/9/2014

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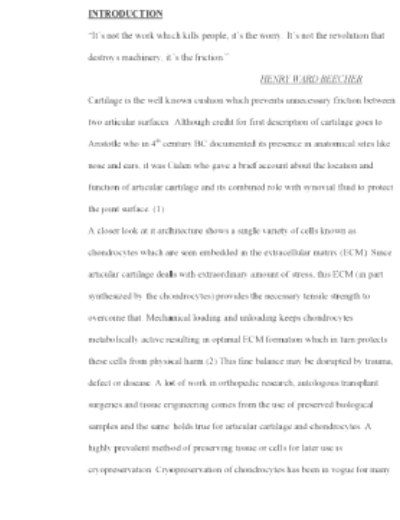

INTRODUCTION

"It's not the work which kills people, it's the worry. It's not the revolution that destroys machinery, it's the friction."

HENRY WARD BEECHER

Cartilage is the well known cushion which prevents unnecessary friction between two articular surfaces. Although credit for first description of cartilage goes to Aristotle who in 4th century BC documented its presence in anatomical sites like nose and ears, it was Galen who gave a brief account about the location and function of articular cartilage and its combined role with synovial fluid to protect the joint surface. (1)

A closer look at it architecture shows a single variety of cells known as chondrocytes which are seen embedded in the extracellular matrix (ECM). Since articular cartilage deals with extraordinary amount of stress, this ECM (in part synthesized by the chondrocytes) provides the necessary tensile strength to overcome that. Mechanical loading and unloading keeps chondrocytes metabolically active resulting in optimal ECM formation which in turn protects these cells from physical harm.(2) This fine balance may be disrupted by trauma, defect or disease. A lot of work in orthopedic research, autologous transplant surgeries and tissue engineering comes from the use of preserved biological samples and the same holds true for articular cartilage and chondrocytes. A highly prevalent method of preserving tissue or cells for later use is cryopreservation. Cryopreservation of chondrocytes has been in vogue for many

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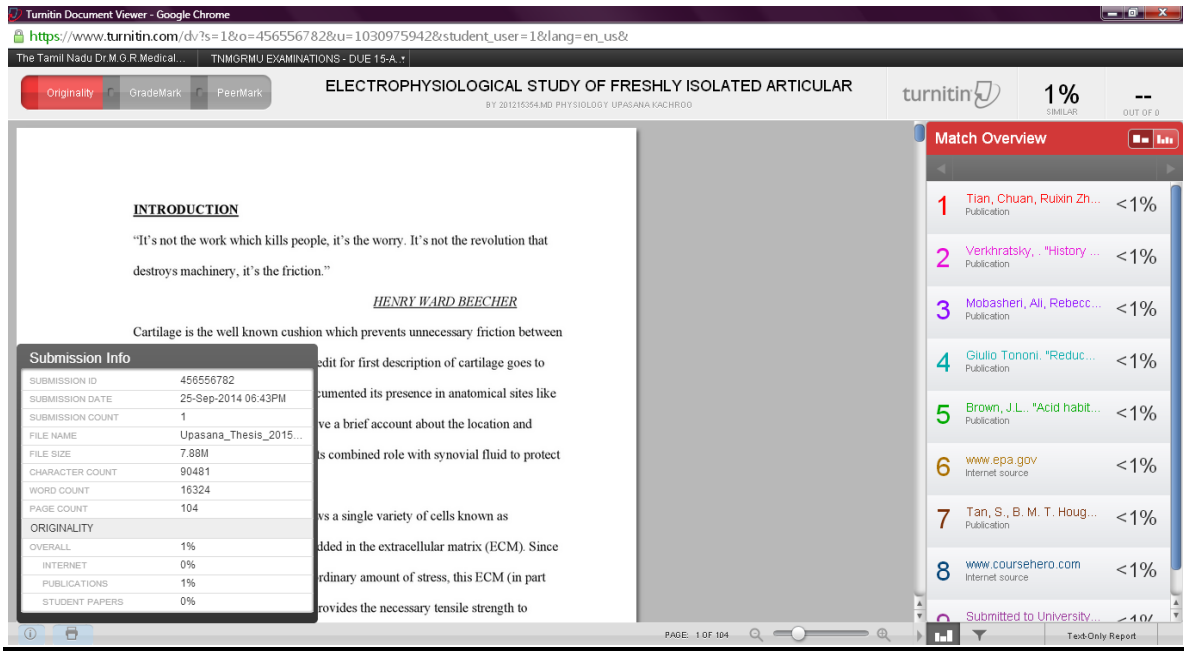
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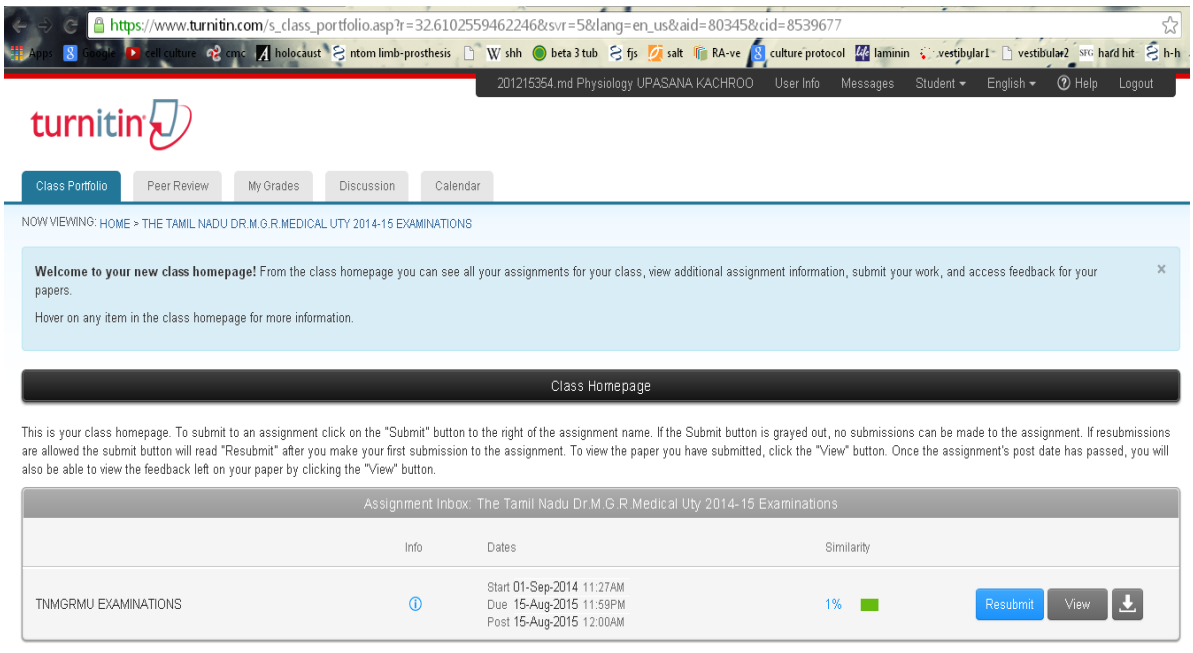
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ABSTRACT

Cryopreservation of articular chondrocytes has recently gained ground because of its applications in cell culture, tissue engineering and reconstructive surgery. Questions may be raised as to the use of cryopreserved chondrocytes since this intervention may cause changes in chondrocyte phenotype and biology. One way of assessing phenotype maintenance is by studying the electrophysiological profile using the patch clamp technique.

This study sought to compare freshly isolated and cryopreserved chondrocytes by recording ionic currents using patch clamp technique. Goat articular chondrocytes were isolated from cartilage shavings by enzyme digestion. Cell aliquots were transferred to liquid nitrogen after overnight gradual cooling to -80° C. Cells were cryopreserved for a period of 7-15 days (2 study groups- Day 7-10 and Day 11-15) Percentage viability was checked upon thawing and cells subjected to patch clamp analysis. Ionic currents were recorded in whole cell configuration using depolarizing potentials (V_{Hold}:-80mV; Test pulses: -80mV to +70mV at 10mV increments).

Outwardly rectifying currents were recorded in fresh chondrocytes (n=6) and in cryopreserved cells (n=9+6) Comparison of current densities at all potentials above the threshold, revealed no significant difference between fresh and cryopreserved chondrocytes ($p>0.05$) Currents were blocked by 10mM TEA^+ (a specific potassium channel blocker). Reversal potential for these currents was observed to be near the calculated equilibrium potential for potassium.

The results indicate that chondrocytes remain viable and maintain ion channel functionality even after 15 days of cryopreservation. Cryopreserved chondrocytes may be utilized as an alternative when the use of freshly isolated chondrocytes poses a limitation.

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years and subsequently cryopreservation of whole cartilage shavings has also been attempted. Many experiments make use of this cryopreserved specimen (preserved cells or cells derived from preserved tissue) under the assumption that there is no phenotypic or functional variation. Even though there are studies which show immuno-histochemical, culture and PCR evidence that cryopreserved chondrocytes behave in a similar manner as freshly isolated cells, it would be prudent to do a detailed analysis. A very sensitive estimation of cell function is by the study of the membrane ion channel profile. This study aims to compare freshly isolated chondrocytes and cryopreserved chondrocytes based on their ion channel profile using Patch clamp technique.

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Articular Cartilage: Physiology

The property of resilience of articular cartilage is courtesy the extra cellular matrix (ECM). This ECM primarily comprises of tension resisting collagens, negatively charged proteoglycans (PGs) which are trapped within the collagen mesh and the interstitial fluid. (3) Proteoglycans by virtue of their negative charge tend to attract cations (predominantly sodium ion) and water thereby causing it to swell. At the steady state, the tension created within the collagen mesh due to proteoglycan swelling causes the matrix to withstand compression. The cationic pull of PGs also favors an external environment of low pH and high osmolarity for the chondrocytes that are residing in the lacunae within the ECM. The matrix to cell ratio found in articular cartilage is very high which means that chondrocytes or the cellular component occupies only about 10% of the entire tissue. (4)

As has been mentioned earlier chondrocytes experience what can only be termed as a somewhat hostile environment as compared to cells elsewhere. Though chondrocytes are resilient enough and adjust very well in this scenario, still drastic changes in the osmotic or ionic environment as seen in degenerative conditions like osteoarthritis may cause significant change in cell function. (3) Effect of alterations in the physical environment can also be seen in an artificial setting when isolated chondrocytes are grown in culture. There are reports of

chondrocytes changing to fibroblastic phenotype while in culture (monolayer). (5)

Articular chondrocytes have been isolated from cartilage and used extensively in cell culture and tissue engineering studies because they have innumerable applications in reconstructive medicine and orthopedic research (defect correction, autologous transplant etc.) (6,7)

Since there is such high demand of specimen, both at the level of tissue and specimen, techniques to preserve the samples acquired from study animal or individual were attempted. A highly prevalent method of preserving chondrocytes isolated from human or animal source (like equine, porcine, bovine) is to freeze them at sub zero levels, a process known as Cryopreservation. (8–10)

CRYOPRESERVATION

It involves techniques or steps used to successfully store cells, tissue, organs or even organ systems under sub zero temperatures without affecting their viability. The efficiency of the technique involved is judged based on how well the biological sample was preserved, both morphologically and functionally.

History

The first successful report of mammalian cell cryopreservation came from Dr. Ernest John Christopher Polge who was able to cryopreserve bull spermatozoa. The temperature of preservation was reported to be -79°C. Dr. Polge and his co-

workers also serendipitously discovered the cryoprotective action of Glycerol which was used as the cryoprotective agent (CPA).(11) The spermatozoa which were retrieved after thawing retained their motility and were used to fertilize ova. Following this breakthrough, various types of cells and tissues were used and cryopreservation became a technique which would have wide application in multidisciplinary research fields.

Basis

Since the major solvent in all biological samples stored is water, therefore the changes occurring in this solvent system due to cooling need to be considered. When water is in the liquid state it dissolves all solute particles but as temperature decreases, ice crystals start to form. These crystals are just pure water molecules and are exclusive of any solutes. The main source of injury to cells is primarily because of this exclusion and the inevitable sequence that follows. Depending on the rate of cooling, ice crystals keep forming leaving a non frozen section which is occupied by solute and cells.

Slow cooling

If the rate of cooling is slow, the high extracellular osmolarity will cause efflux of intracellular water causing cell shrinkage. As cooling continues, the viscosity of the non frozen fraction keeps increases to a point where further ice formation has to stop. This fraction appears as an amorphous solid without any ice crystals.

Rapid cooling

In case of rapid drop in temperature, cell shrinkage is comparatively less as water does not get sufficient time to move out of the cells but at the same time it creates another problem. All the water trapped with the cell forms ice crystals and causes damage. Recrystallization while thawing also poses a problem with this technique. Cell permeability is a factor that affects water movement. More permeable cells may tolerate rapid cooling better while slow cooling is a better option for less permeable cells.

Cellular injury

The main challenge faced by cells when temperature is reduced to such low values way beyond the homeothermy they usually experience is direct damage due to fast cooling-cold shock and the direct effect of the extremely low temperature- chilling injury. The functional properties and behavior of cells is also altered because the temperature directly affects the membrane lipids and proteins. The solidification of these components can potentiate events like production of reactive oxygen species which will cause further damage. Cellular metabolism and other processes are also affected owing to low temperature. One example of this effect is disintegration of spindles formed during metaphase in oocytes due to disturbance of equilibrium between association/dissociation of tubulin filaments.

Supercooling and Unfrozen Fraction

When the sample is cryopreserved and temperature reduced, it experiences sudden and huge temperature changes. This is explained by the fact ice formation and the freezing point may not necessarily coincide. Ice crystal formation occurs at a lower point that bulk ice formation owing to the higher surface tension. Supercooling is reducing the temperature of a liquid (here) below its freezing point without turning it into a solid. For water, spontaneous ice nucleation starts after supercooling reaches a temperature of -5 to -15°C. Once this happens, there is ice formation in all regions which causes release of latent heat of phase change bringing about abrupt temperature change which is corrected only when the freezing point of the rest of the solution is reached.

The very high solute concentration encountered by cells in the unfrozen section leading to dehydration and shrinkage may cause membrane instability and protein denaturation. The water movement can be so extreme that cells may lose upto fifty percent of their original volume leading to deformation of cell structure. Cells also experience mechanical stress due to confinement in the unfrozen section which is small and continually impeded by ice crystals being formed.

CRYOPROTECTIVE AGENTS (CPA)/ CRYOPROTECTANTS

In practice, at any cooling rate, the subzero temperature is the main factor determining the total solute concentration. Initially when the cryopreservative medium contains only electrolytes, salt concentration peaks rapidly as

temperature decreases. On the other hand, in suspensions containing non-electrolytes, even though the total concentration of solute is equal to that of any other suspension (at that temperature) having just electrolytes, but the salt concentration is comparatively much lower.

Sugars are common non-electrolyte which can be used (were tried). The problem they pose though is that they create an environment of high osmolarity outside the cell since they are impermeant. This increases the stress on the cells even before the freezing has actually begun.

This is where the role of a better cryoprotectant comes into play. It is basically a permeant non-electrolyte eg. Glycerol (first CPA used). When the sample is suspended in medium containing substantial amount of glycerol, there is initial efflux of intracellular fluid which results in cell volume loss. But at the same time, glycerol is entering the cell, and after what is known as Equilibration Time, cells regain their volume. This means that in comparison to solutions containing sugar, the ones containing concentrated glycerol may be used as the amount of osmotic stress experienced by the cells would be much less in case of glycerol. Since it is also a permeant non- electrolyte, a substantial amount of intra and extracellular space is taken up by this solute replacing water. The direct result of this is less space for ice crystal formation, bigger unfrozen section, less cellular shrinkage and less salt concentration in the unfrozen area. These effects primarily make up the main functions of any cryoprotective agent.

Types of CPA

Cryoprotective agents can be of two kinds:

- Penetrating or permeant like Glycerol, Dimethyl sulfoxide (DMSO), Ethylene glycol
- Non-penetrating or impermeant like Polyethylene glycol (PEG) , Polyvinylpyrrolidone (PVP)

Permeant agents are small sized molecules that function in two ways. They reduce the amount of cell shrinkage and intracellular crystallization by entering the cell. Also they keep the unfrozen section relatively bigger so mechanical stress is reduced. On the other hand, non-penetrating agents are bigger molecules which cannot cross cell membrane and therefore are only helpful in increasing the unfrozen area.

The amount of cellular shrinkage and swelling experienced after addition of the cryoprotective agent and lowering of temperature is dependent on the concentration of cryoprotectant used and also the relative membrane permeability to water and the non-electrolyte. (12) Another CPA related challenge faced is during thawing, when the cells go through phases completely opposite to the ones happening during freezing. On removal of the cryoprotective agent the cells initially swell and then shrink back. Excessive swelling may cause damage to the cells which have already undergone cryogenic stress. This may be reduced by step by step removal of the cryoprotectant. It is known that while freezing, substantial cellular water volume is replaced by the cryoprotectant.

Although that helps prevent injury by crystallization but it creates a new problem of cryoprotectant toxicity. The exact mechanism of induction is not clear but lipophilicity has strong co relation with toxicity. Lipophilic CPAs with affinity to fat molecules may cause membrane partition and its destabilization. There are also reports suggesting that toxicity may be strongly related to hydrogen bonding probably because of affection of the hydration shell of certain macromolecules. Cell membrane toxicity has also been related to the electrical properties of the cryoprotective agents and solutions used.(13)

Cooling & Thawing Rates- Interaction with CPA

When any biological specimen or cell needs to be cryopreserved, its optimal cooling rate should be assessed and used as per protocol. Survival rate if adversely affected if the cooling rate is low (causing slow-cooling damage) or very high (causing fast-cooling damage). (14) The cellular membrane acts as a resistive barrier to water movement hence slowing its transport. In comparison the formation of ice happens relatively faster. As already explained, although slow cooling offers the cells a chance to rid themselves of intracellular water thereby reducing intracellular crystallization, but at the same time these cells suffer more osmotic stress. Conversely, rapid cooling reduces osmotic stress by reducing the time for dehydration but increase the chance of damage by intracellular ice crystal formation (by the water lag). Other proposed mechanisms of injury by this method are uneven pressure distribution on the membrane due to rapid efflux of water and changes in cell size and ultrastructure.(15) Factors that

determine optimal cooling rate for any biological specimen or cell would primarily be cellular volume, (membrane) surface area, and how permeable the cell membrane is to water and the cryoprotectant. Other factors which may influence optimal cooling rate are concentration of cryoprotectant used and the rate of thawing of the specimen. The detrimental effect of slow thawing and rapid cooling has been demonstrated in various cells. If cooling is very fast and ice nucleation happens at a low temperature then by the time ice crystal size reaches a considerable amount, intracellular space would have become amorphous leading to little damage to cell. However on thawing, which proceeds slowly, crystal formation starts and spreads leading to extensive cellular injury. Also cells are damaged by Recrystallization, a phenomenon where the ice crystals formed in the extracellular space undergo restructuring.

Freezers in Use

Depending on the financial resources available to the laboratory planning to cryopreserve biological specimens, freezing can be done using programmable or simpler, non-programmable freezers.

In programmable freezers, the extremely cold nitrogen vapour is used to bring down the temperature of the cryovials. A program may be generated to regulate the temperature in the cooling container and also control the cooling rate. But controlling the sample temperature is not easy due to warming caused by release of heat of fusion. In case of non-programmable freezers, vials are cooled by exposing them to vapour of a cold surface which will provide a low temperature

constantly. An example of a simple non-programmable freezer would be a Styrofoam box without ventilation which holds a rack of specimen filled vials partially filled with liquid nitrogen. In such a setup, the amount of heat exchanged depends on the temperature difference existing inside and outside the specimen holder and also on the rate at which heat is being conducted. Rate of conduction of heat depends on volume to surface ratio of the vial being used and ventilator rate (forced). It therefore required repeated experimentation to obtain optimal cooling rate using any non-programmable freezer. However the results obtained can be as good as the ones given by any commercially available automated freezer. However an advantage that using a non-programmable freezer gives is a more beneficial cooling curve which is the time course involving cooling and freezing. The curve predicted in theory to be optimal for slow cooling is what is nearly achieved when non-programmable freezers are utilized. The trend follows the path of a slow cooling rate right after ice formation and faster cooling rate in the period thereafter. Majority of the ice forms between the freezing point and -10° to -15°C. Also, maximum water movement out of the cell occurs at the same time. Latent heat that is released during phase change increases the temperature of the suspension and affords the cell enough time to conveniently transport intracellular water to the extracellular space. Therefore by adjusting the height at which the vial will be stored in the non-programmable freezer, one can adjust how steep the cooling curve will be. In case of

programmed freezers, the curve can be controlled by changing the temperature of the vapour or the ventilation.

VITRIFICATION

The meaning of this term is transformation to glass. Here the liquid to solid transition occurs but is devoid of any ice crystal formation. This is highly beneficial since cells can escape injury caused by crystallization. The question arising here is how slow cooling a specimen is different from vitrification. The answer to that lies in the fact that for vitrification, the concentration of non-electrolyte used is very high causing almost nil ice formation. As the amount of ice being formed is very low, cooling need not be slow and infact would be more beneficial if rapid. The final state of glass like quality achieved and the physico-chemical condition that vitrification or slow cooling produce is almost alike but the methodology involved to reach this point varies.

Role of CPAs

The biological specimen is generally introduced into a solution containing very high concentration of CPAs. If the concentration is adequate, the vitrification solution will turn into an amorphous solid without any crystallization occurring either while cooling or thawing of the sample irrespective of the cooling or re-warming rate being used. The flipside of using such high concentrations is that cells experience high degree of osmotic stress and direct toxicity from the cryoprotectant being used. It is advisable therefore to increase cryoprotectant concentration in a step wise manner with equilibration at room temperature

followed by increase in concentration at a lower temperature followed lastly by quick transfer to the freezer. This helps reduce osmotic stress while the cryoprotectant induced toxicity is controlled by the reduced temperature and rapid cooling. Another method to reduce toxicity is to use a solution containing both permeant and non-permeant cryoprotective agents since latter donot enter cells but just form part of the extracellular unfrozen fraction. Examples of non-permeant CPAs that have been used in combination for vitrification are polyethylene glycol or bovine serum albumin (60grams/litre) (16) If the suspension containing the sample has CPA concentration less than that of standard vitrification solution then there is a possibility of ice crystal formation while cooling. But at the same time a cooling curve that is very steep does not allow water enough time to even form ice and converts the whole solution to an amorphous state directly also called metastable glass like state. As the cooling curve becomes steeper the cryoprotectant concentration required to achieve this state reduces which thereby decreases solute induced osmotic damage as well as chemical toxicity.

Various ways to increase cooling rate are firstly to reduce the sample volume. Smaller the volume to be vitrified, easier it is to cool the solution rapidly. Volumes as low as 0.1 microlitres are under use.(17) The second method that may be employed to increase cooling rates is transferring heat to a non-boiling liquid. Liquid nitrogen on absorbing heat will finally turn to nitrogen gas at its boiling point. This gas forms a coat of insulation around the sample vial and

reduces contact with cold liquid. On the other hand, at its freezing point liquid nitrogen forms what is called Nitrogen Slush which is devoid of this problem. Special apparatus is required to produce nitrogen slush.

One precaution to be observed while trying to achieve metastable vitrification is that thawing or re-warming of the sample should be rapid. If thawing is delayed or gradual, ice crystal formation will start when the temperature between the vitrification temperature and the freezing point of the solution is reached.

Cryopreservative solutions- general considerations

This solution may be used for either directly freezing the sample or for vitrification. The main components are:

I. Carrier solution- it has constituents that are not precisely cryoprotectant solutes. It provides balance to the specimen at near freezing temperature and contains molecules like electrolytes, pH buffers, nutritive agents and inhibitors of apoptosis. The concentration of solute in this solution is kept iso-osmolar. Even though cryoprotectant concentration sequentially increases during vitrification, the composition in carrier solution remains constant and therefore should be prepared carefully.

II. Cryoprotective agent- As already discussed this forms a very important part of the cryoprotective solution. Penetrating CPAs have the task of preventing ice crystal formation therefore they form a major component of cryoprotective solutions. Non-penetrating CPAs help by reducing the requirement for permeant

CPAs as they perform the same cryoprotective function as the non-electrolyte solutes within the cell.

III. Ice blockers- these are not routine ingredients and are complimentary to CPA function. These molecules prevent further ice formation by directly binding to ice or to the molecules acting as ice nucleators. These agents are conventionally not present in freezing solutions but are a part of many vitrification solutions.

FREEZE DRYING

Another method which has been employed to preserve biological specimens is freeze drying because of its cost efficiency. It negates the need for cryogenic liquids and expensive storage equipment. Since the samples may be stored at ambient temperatures, it also proves to be a safer option as injuries occurring as a result of hardware malfunction and cryogen spillage are prevented. The biggest drawback of this technique is a reduction in cellular viability. The main principle involved in freeze drying is to bring the sample to an amorphous state where the glass transition temperature is more than the preferred storage temperature which is generally ambient. Part one of the technique involves vitrification of the sample. Next, this vitrifies specimen experiences vacuum due to which the ice crystals present undergo sublimation thereby reducing the water content of the sample considerably. This solvent removal causes an increase in the glass transition temperature which rises above ambient temperature. The sample can

therefore be stored conveniently although the steps required to vitrify the specimen should be optimized as it will experience stress due to additional dehydration. Since cell viability on retrieval is a prime factor while cryopreserving certain types of cells or biological specimen therefore cryogenic liquids like Liquid nitrogen are still extensively used.

LIQUID NITROGEN

Nitrogen gas forms about 78% of the total atmospheric gas volume. It is an inert, non-inflammable gas which does not support life. When Nitrogen gas is liquefied it turns to an extremely cold, colourless, odourless, non- corrosive state which is immensely useful in cryopreservation.

In general cryogenic liquids are gases which have been liquefied and have a boiling point below -150°C. Boiling point for liquid nitrogen has been seen to be about 195.8°C. Like other cryogenic liquids, it also tends to produce large amount of gas when it vaporizes.

Handling care and hazards:

Detrimental effects arise due to quick evaporation and the very low temperature of the liquid. Common hazards that are encountered are:

- Asphyxia

The volume expansion that liquid nitrogen undergoes is about 680 times when is vapourizes. Due to this quick and immense increase in volume, it can easily displace the oxygen present in the environment especially in small, confined areas which are not well ventilated. The degree of asphyxia depends on the reduction in oxygen volume in the ambient environment. If levels reduce to about 18%, noticeable asphyxia occurs. A reduction below 6% may cause death. The condition worsens as there are no immediate warning signals which might alarm working personnel since the gas is colourless, odourless and inert. Any indication of oxygen deprivation generally occurs very late and is also masked by impaired judgment and ability to perceive for the victim to be able to respond quickly. The result is unconsciousness and in extreme cases death. It becomes all the more important in this case, that rescuer not enter the chamber unless trained and equipped with apparatus to breathe safely.

- Cold/Cryogenic Burns

Since the temperature maintained for/by Liquid nitrogen is extremely low, any direct contact with the liquid or non-insulated parts of the container holding the liquid may cause serious tissue injury. The effect observed when exposed skin

comes in contact with the liquid is similar to a burn with prolonged exposure causing frostbite. Initially tissue may show discolouration in the form of grey or grayish-yellow patch which whereas frost bitten tissue may go become non viable if not treated promptly. Delicate areas like eyes are more susceptible to damage while inhalation of large amount of vapor and mist may cause lung injury. Skin contact with container or parts of it may cause cryogenic burn or adherence to the non-insulated equipment itself. Any attempt to rapidly or forcibly withdraw the body part can result in tearing of flesh. Use of appropriate protocol and wearing protective clothing can minimize these risks. First aid advised in case of injury sustained is:

a. Distancing the individual from the source and taking to a warm area

b. Using tepid water to flush the skin and to seek medical help

c. Preventing further injury by avoiding strong jets of water

d. Avoiding application of heat or hot water to affected area

e. Removing tight clothing, articles of jewellery carefully

f. In case of severe burns, individual to be rushed to hospital in an ambulance

- Pressure build up

In order to discuss this effect, some knowledge is required about the storage devices used for these liquids. There are different kinds of units used for

transportation, handling and storage of liquid nitrogen. The choice of container depends on the quantity of the liquid to be used. They are:

a. Dewars: These containers are non-pressurized and the capacity ranges from 5-200 litres. The samples are either poured out or in the case of bigger containers, a transfer tube is utilized

b. Cylinders: These are insulated pressure containers with vacuum jackets. They have additional safety valves and rupture discs to relieve excess pressure. The capacity ranges from about 80 to 450 litres and it can work at pressures upto 350 psi.

c. Storage tanks: Shape of the container may be cylindrical or spherical. They are generally kept stationary in a designated space. Most of these tanks are custom designed to fit temperature and pressure guidelines.

When dewars are being used, there is possibility of ice plug formation at the neck of the flask which has been left open. Extreme caution should be practiced in such cases as the outlet may get blocked in such cases leading to pressure build up in the container. This may cause the ice plug to be propelled explosively or even possible rupture of the container itself. To prevent ice plug formation suitable dewar stoppers should be routinely used. In case of plug formation, pressure may be relieved by piercing a hole through the ice by using a heated L-shaped wire. This should only be done wearing appropriate clothing and complete facial shield.

Cryovials or microcentrifugation tubes used to store samples can sometimes explode when retrieved from the container. This happens when some liquid nitrogen enters the tube while it is stored in the container. The probability of liquid nitrogen entering the vials increases when they are stored in the liquid phase and not in the vapour phase of the storage container. Precautions to be taken are either encasing the vials in an outer container to protect them and letting them thaw in this manner till desired temperature is reached. Also for sample retrieval, protective eyewear and gloves should always be used.

- Oxygen enrichment

Atmospheric oxygen may condense and liquefy owing to the cold temperature in the confined area created by the cryogenic liquid. This liquid oxygen can prove to be very dangerous as it is highly combustible and increases the risk of explosions and accidental fires. Oxygen enrichment becomes a threat when dealing with closed systems, since any air leak may cause condensation of oxygen and further contact with ignition systems or unstable chemicals may result in catastrophes.

- Material friability and Embrittlement

Due to the extremely low temperature of the liquid, care should be exercised while selecting container material to transport and store liquid nitrogen in the lab. Spillage from the container onto the floor may cause damage to certain surfaces like vinyl, making them brittle and therefore unsuitable in the lab as they can cause slipping hazard.

HANDLING

Containers meant for transport or storage of the liquid should always be carried or installed in an upright manner with minimal agitation. Any kind of thermal or mechanical disturbance should be avoided and the container should not be left unattended if being transported. While transferring the liquid, it should be made sure that the flow is directed below the mouth of the receiving vessel.

Warm containers should be filled in a slow manner to prevent splashing and thermal shock. Also cylinders and dewars should not be filled beyond an 80% capacity.

The area of storage should be ventilated according to the container and amount of liquid in use. For small containers, passive ventilation from the room is generally sufficient but this may not be the case if larger vessels are being stored or if leak causing huge amount of gas release is possible. In cases like these, additional measures must be taken or accessory vents provided. Periodic assessment of liquid level in containers and record of top up volumes should be made to make sure that evaporation rate is almost constant. Any major deviation may be an indicator of faulty container body or deteriorating insulation or valve mechanism.(18)

It is advisable to display safety guidelines and usage protocol for all users and non- users to minimize hazards and promote safe usage.

As can be seen, the temperature conditions prevalent in the container holding the cryogenic liquid and the physical and chemical stress imposed by the CPA wmay cause the biological sample to undergo certain changes while in storage. Same is true for cryopreserved chondrocytes. Though this class of cells is known for its resilience and ability to adjust to changes in physical environment, questions could be raised as to the quality of cells that were retrieved after cryopreservation. To answer these doubts many studies were done of which some compared the replicative potential of cryopreserved cells in culture versus fresh chondrocytes by comparing the doubling time. (19) Another study used human articular chondrocytes and compared Collagen II expression in cryopreserved and freshly isolated chondrocytes.(20) Another study made use of equine chondrocytes and compared performance in monolayer and 3D culture. (10) There are also reports where work has been done on cryopreserved chondrocytes and freshly isolated cells side by side, probably under the assumption that cryopreservation does not cause any functional change in these cells. This includes electrophysiological studies done on human articular chondrocytes, both freshly isolated as well as cryopreserved to look for the presence of membrane ion channels (potassium channels) (21) Although these studies suggest that irrespective of drop in viability on retrieval, cryopreserved chondrocytes show more or less the same phenotypic and functional characteristics as freshly isolated cells, it would be beneficial to future research involving their use if focus is given to investigate the effects of cryopreservation on these cells. (6,10,20–23)

A very sensitive analysis comes in the form of electrophysiological study of the membrane ion channel profile which can get altered by extreme changes in physical environment as is seen in cryopreservation.

ELECTROPHYSIOLOGY

It is the branch of physiology which deals with the study of electrical properties of biological specimen in the form of whole organs, tissues or cells. It encompasses all the techniques that involve recording of electrical phenomena arising due to ionic flow. Most commonly the setup involves placing electrodes over/into various sites of a prepared biological sample. Electrodes used primarily are:

I. Needle or disc electrodes (solid conductor)
II. Electrolyte solution acting as electrode when filled into a hollow tube like a glass pipette
III. Traces made on circuit board which are printed

The specimen may be prepared in the following way:

i. Cells or tissues grown artificially
ii. Cells teased from tissues or excised organs
iii. Organ or tissue after excision
iv. Organism
v. A mix or hybrid of the types mentioned above

HISTORY & DEVELOPMENT

In late 1600s, Dutch microscopist by the name of Jan Swammerman developed a preparation using a frog's thigh muscle with intact nerve supply. When the nerve was stimulated, the muscle contracted. He further improved his experiment by introducing the specimen into a hollow tube made of glass while attaching needles to the ends of the muscle. With this preparation where 'irritation' (as Swammerman reported it) of the nerve led to contraction of the muscle, the basic foundation of experimental electrophysiology was laid. (24) 80 years later, in 1791 Luigi Galvani gave experimental support by publishing his work on what he termed as animal electricity. The preparation used by Galvani consisted of frog lower limbs connected by crural nerves to the spinal cord under full exposure while a metal wire passed through the vertebral canal. Using stimulation of the nerve Galvani was able to identify and demonstrate electrical excitation. He was also able to establish a relationship between intensity of stimulus given and contraction of the muscle achieved which as he observed by repeated experiments showed saturation. He noticed that increasing the intensity of stimulation beyond a certain point did not result in increase in strength of contraction. Also, stimulation given on a repeated basis caused contraction to stop which could be revived on giving the tissue a while to rest. Based on this observation, Galvani proposed the refractory phenomenon.(25) He was also the first to demonstrate action potential propagation in his preparation where two

frog legs were used with attached sciatic nerves. When the sciatic nerve from one leg was in contact with the muscle or nerve from the other leg, contraction was seen in both preparations. All the experiments done by Galvani made him postulate the Theory of electrical excitation. According to him, biological tissue could generate electrical signals in response to the external stimuli even at rest because it was in a state of disequilibrium. He also believed that accumulation of charges, positive and negative- both on either surface (external and internal) of tissues like muscle and nerve result in animal electricity. (25)

Instruments in electrophysiology

Using an electromagnetic galvanometer, Leopoldo Nobili became the first man to make an instrumental recording of what Galvani called animal electricity using a neuromuscular preparation from frog leg.(26) Hermann von Helmholtz was able to determine speed of propagation of nerve impulse in 1850-1852 using nerve muscle preparation. Using a smoked drum for the first time to record muscle contraction, he was able to measure the delay between electrical stimulus application and contraction of the muscle. Later Julius Bernstein, who has been credited with the invention of differential rheotome, became the first ever to record what are known to be true resting membrane and action potentials. Bernstein's student Vassily Tschagovetz in 1896 was the one to propose that excitable membrane's potassium ion selectivity was the cause for generating resting membrane potential. (27) It is interesting to note that for Tschagovetz to

formulate his hypothesis, he applied the electrolytic theory proposed by Walther Nernst to biological systems. In addition to further developing what had already been put forth by Tschagovetz, Charles Ernst Overton made substantial contribution to membrane physiology by proposing that it was exchange of sodium and potassium ions which caused the excitation process. He was also the first person to suggest that plasma membrane was primarily "lipoidal". Danielli and Davson later confirmed and developed this theory further by the introduction of a concept which suggested that plasma membrane was composed of a lipid bilayer with which many protein molecules are associated. Numerous water filled pores permeate the membrane and allow many molecules to pass through them including various lipid-insoluble structures like ions.(28) In this way, somewhere by mid-1930s, ionic channel prototypes had been suggested.

Voltage Clamp

Direct experiments pertaining to electrophysiology were first done using squid axon and were introduced by John Z. Young. A little after 1939, Kenneth Cole and Howard Curtis who had been performing experiments to measure impedance and also Alan Hodgkin and Andrew Huxley were able to perform experiments and directly record action potentials by developing and introducing intracellular electrodes into the squid giant axon.(29,30). Credit for design of voltage clamp technique goes to Cole and Marmont who introduced it in 1949, but it was the team of Hodgkin and Huxley who took it forward by a leap as they utilized a

dual electrode approach for potential measurement and also performed series resistance compensation. These modifications helped them directly record current flow across axonal membrane (squid giant axon) owing to ionic movement. Aided by Bernard Katz, they were also able to look at the voltage sensitivity and ion channel kinetics.(29) Their extensive and laborious work on understanding membrane physiology, led them to formulate what is now known as the Hodgkin-Huxley mathematical model of action potential. This model was proposed to explain the kinetics of the currents studied in their experiments. The model was denoted by 'm^3h, n^4' where n stands for open probability for each charged particle (potassium ion), m was activation of sodium current, fitted by three charged particles, and h denoted inactivation of sodium current. This model constituted of mainly four currents. They were:

a. Sodium current
b. Potassium current
c. Capacitance current
d. Leak current

The accuracy with which time course of the action potential could be predicted using this model was remarkable. After their pioneering work together, Hodgkin continued working on Squid axon to study and investigate Calcium and Sodium pump while Huxley's field of interest shifted towards mechanisms involved in activation of contraction of skeletal muscle.

Ion channel recording & Patch Clamp

There were a lot of roadblocks faced when making recordings from ionic channels which needed to be overcome. Firstly, the size of cells itself posed a very big problem as mammalian cells were very small in comparison to the giant axon isolated from the squid. Also isolation of intact cells from the tissue itself proved difficult. Second, to be able to record clean currents and also separate the regulatory mechanisms that modulated the current, one had to have extreme control over the intracellular as well as extracellular environment. Third challenge in attaining good recordings was the noise reduction required from extremely small fractions of cell membranes. First account of intracellular recording comes from the work of Gilbert Ling and Ralf Gerard who were the ones to introduce microelectrodes made by pulling glass pipettes in 1949.(31) Improvements in the technique to record membrane currents and reduce noise using extracellular micropipettes were brought about by a number of electro-physiologists over the years where modifications such as suction to the micropipette, intracellular perfusion and placement of microelectrode. It was the team of Erwin Neher and Bert Sakmann who, by introducing the technique of patch clamp provided the solution to a major problem faced by many while recording ionic currents. (32) Background noise was a limiting factor as it made it very difficult to detect current from a single channel while an intact cell was being studied. Conventionally, when an intracellular micropipette was used, background noise of the order of around 100 pA was generated. The amplitude of

current from a single ionic channel was very small compared to this noise. This problem was overcome when Neher and Sakmann decided to use a fire polished micropipette with a smooth tip to the surface of a muscle fiber of the skeletal type. This caused electrical isolation of that patch of the plasma membrane. Since the amount of noise decreases as the area under study is reduced therefore by being able to segregate only a fraction of the membrane for study they were able to reduce the noise so much that ionic current recordings through single channels could be made directly.(32) There was another limitation in the form of low seal resistance (at the junction of the micropipette and the cell membrane in contact) which could affect the quality of the recording. This was remedied in 1980 with the discovery of the technique to attain a seal with a high resistance between the recording pipette and the membrane in question. The seal resistance attained was of the order of gigs ohms and therefore came to be widely known as Gigaseal. The recordings made using this method were much more stable and cleaner since mechanical isolation as well as electrical isolation of the membrane patch was now possible.(33)

Patch clamp-Variations

While using patch clamp to obtain recordings from cells, the position of the micropipette and the area of the membrane subject to examination along with the solutions (bath and pipette) prepared to control intra and extracellular environments give rise to various kinds of configurations that may be used while

patching different types of cells or while investigating different kinds of ion channels. Commonly used patch clamp configurations are (Figure 1):

a) Cell attached mode: this is the first configuration that may be obtained because herein the pipette and a part of the entire, intact cell have formed a seal. In this configuration, the patch surface membrane potential can be controlled relative to cellular resting potential. This technique is beneficial as the entire cell remains unharmed and therefore one can maintain the intracellular environment. Also certain channel modulators which may have intracellular mechanisms may be studied without disturbing the cell. Eg. Calcium channel- system of intracellular second messengers. One important factor to be aware of while recording in this configuration is that both the seal as well as the positioning of the pipette with respect to the cell should be stable.

b) Whole cell & perforated patch mode: in both the configurations, the type of macroscopic current recording from the cell is almost similar but they themselves are quite different. In case of whole cell mode, a patch of the membrane already sealed (cell attached) is broken into, either by application of mild suction or by using a large amplitude voltage pulse applied briefly through the micropipette. The method used to break into the cell is dependent on the cell itself. Voltage pulse is used for cells where suction causes loss of seal instead of gaining electrical access. As soon as patch disruption has taken place, current clamp may be used to directly measure the membrane potential or using voltage clamp, membrane potential maybe controlled. While recording via this mode, pipette

solution dialyses cell contents and one can also introduce various compounds into the cell. It should be noted that equilibration of intracellular fluid and pipette solution is limited by the pipette tip and therefore may prove to be a hindrance while investigating channels involving active transport. Eg. Sodium potassium pump.

Perforated patch mode on the other hand (introduced in 1988 by Horn and Marty) uses a modification involving the micropipette. The tip of the pipette is first dipped into routine pipette solution (called Tip dip) but the filling of the rest of the pipette is done using pipette solution (Back fill) to which compounds like Nystatin have been added. Amphotericin is another antibiotic which has been used. Duration for which tip dip is done depends on fabrication of pipette and what resistance it yields. The pipette solution containing the antibiotics is always sonicated before use and ideally should be used within 2-3 hours of preparation. Once the solution has been filled, Giga seal should be obtained as soon as possible (before nystatin reaches the tip). After the antibiotic reaches the tip, it permeablizes the membrane by insertion into the membrane (Resistance reduces slowly and capacitance increases). This method is advantageous as the pores created by Nystatin are big enough for monovalent ions but impermeant to divalent ions. They do not disturb intracellular calcium ion concentration and hence recordings made are better controlled than whole cell mode.

c) Outside out patch & Perforated Vesicle: In order get current activity from a single channel and record it, these modes may be used. Here bath solution is

carrying the compounds or modulators under study, bathes the external surface of a small fraction of the cell membrane which is used for patch clamp studies. The outside-out patch of membrane is obtained by initially attaining the whole cell mode. Similarly to get to the perforated vesicle, perforated patch configuration needs to be attained first. The volume of cytoplasm held inside the perforated vesicle varies. Also due to the pore forming property of antibiotics used in perforated patch, the extent to which compounds may be introduced into the cell is limited.

d) Inside-out mode: This configuration is used to attain recordings from single channels and therefore only small area of membrane is utilized. It differs from outside-out patch as in this case the intracellular surface of the membrane is exposed to the bath solution. One limitation of using this mode is potential channel activity wash out. But by perfusing the intracellular side of the cell, many investigations may be carried out. Eg. How a compound affects channel inactivation or how another molecule may alter run down and reverse it. Unlike perforated vesicle, pipette perfusion is possible in this configuration. The stability of the seal is also remarkable.

e) Loose patch configuration: In case of tissues where attaining a stable, tight seal between the recording-pipette and the membrane is not possible, this configuration may be used. Analog compensation is used with the help of commercially available amplifiers to make up for the poor seal. Improved forms include using concentric electrodes instead of a recording pipette which

generates potential gradients. One pipette is used to make several recordings. The objective is to map current recordings from large cells. Since the cell is not invaded, intracellular mechanisms are not disturbed and therefore this configuration is slightly similar to cell attached mode in this respect. Recordings are representative of a large number of channels as the diameter of the recording electrode is wide. Membrane potential just like in cell attached mode needs to be measured separately or should be estimated independently.

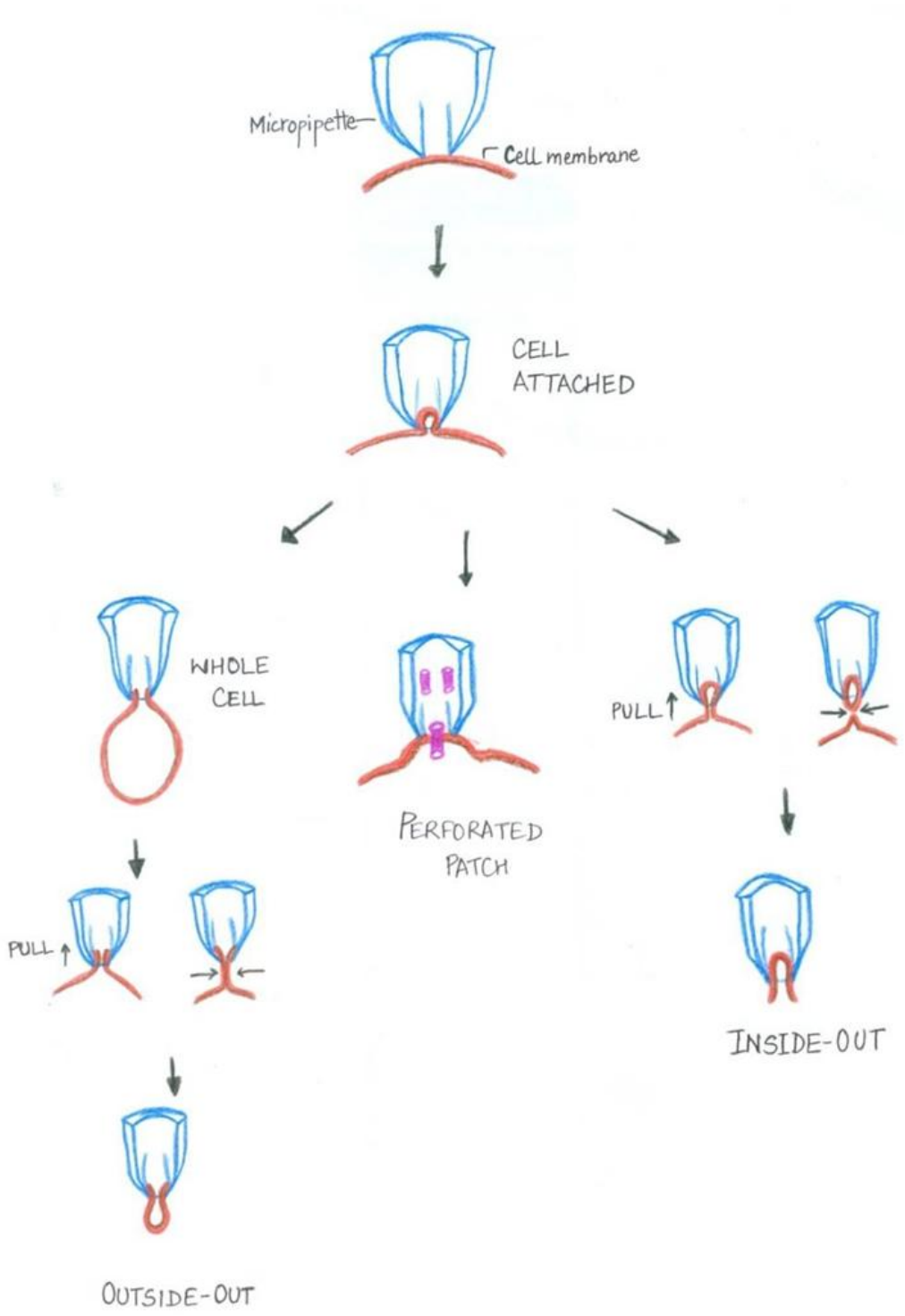

Figure1. Diagrammatical representation of various Patch clamp configurations in use

ION CHANNELS DESCRIBED IN CHONDROCYTES

There have been numerous studies which have used patch clamp to describe the various ionic channels present on chondrocyte membrane or what is known as the chondrocyte channelome. Presence of the following channels has been demonstrated in chondrocytes till date in various studies using patch clamp technique and immuno-histochemistry:

Potassium channels: (explained in detail later) (2)

a. Voltage gated potassium channels
b. Calcium activated potassium channels
c. Inwardly rectifying potassium channels (K_{ATP} subtype)
d. Tandem pore potassium channels

Transient receptor potential channels (TRP):

It is a family of channels which shows less selectivity for ion permeability among sodium, calcium and magnesium ions. Presence of TRP channels in osteoarthritic cartilage was confirmed by PCR and specifically TRP4 channel has been found in canine and porcine chondrocytes. This channel is believed to be stretch activated causing calcium ion entry and has also been related to chondrogenesis and volume regulation.(34)

Voltage gated calcium channels (VGCC):

Presence of L-type VGCC has been suggested following ultra-structural studies in mouse limb bud chondrocytes but the data needs to be supported as in other

studies, intracellular calcium concentration change influenced by osmotic stress was not influenced by the use of Verapamil which is an L-type calcium channel blocker. (35,36)

Voltage gated sodium channel:

Presence of VGSC sensitive to Tetrodotoxin has been reported in rabbit chondrocytes and in osteoatrthritic cartilage. But the literature finds little support from other studies.(37)

Epithelial sodium channels (ENaC):

There is both electrophysiological as well as immuno-histochemical evidence for the presence of ENaC in chondrocytes.(38) This channel is a part of the Degenerin and ENaC superfamily. A heteromeric channel, it may constitute upto 4 subunits: α, β, γ and δ. In chondrocytes α and β sub units have been demonstrated by immuno-histochemistry. The known blocker for this channel type is Amiloride and the proposed function for this channel in chondrocytes is that of mechano-transduction (contributing to RMP maintenance).

Chloride Channels (ClC):

First described in Xenopus oocytes, chloride channels have since been reported in many cells including chondrocytes. Studies have shown the presence of ClC in rabbit articular chondrocytes and in a human chondrocyte derived cell line (OUMS-27).(37) Using channel blocker SITS, (4-acetamido-4′-isothiocyanatostilbene-2, 2-disulfonic acid) it was demonstrated that this channel

was important for controlling resting membrane potential and also for chondrocyte survival. The only subtype of chloride channels identified so far at the molecular level in chondrocytes is the CFTR (cystic fibrosis transmembrane conductance regulator).(39) CFTR functions as an individual channel as well as regulates the activity of some other membrane channels.

Aquaporin Channels:

(AQP) Aquaporins are a family of small sized proteins integrated into the membrane and have been related to MIP (major intrinsic protein) or AQP0. Classical aquaporins are exclusive transporters of water but there are few small, uncharged molecule carrying aquaporins namely AQP3, AQP7, AQP9 (carry molecules like urea and glycerol). Presence of aquaporins has been demonstrated in chondrocytes and their function studied by inhibiting their activity reversibly using mercuric chloride. Proposed role of aquaporins in chondrocytes is volume regulation and also cell migration and adhesion.

Other channels:

The more recent literature reports a few more channels in chondrocyte membrane of which ASIC (acid sensing channel) and Connexin 43 hemichannel are newer additions.

ASIC is a channel type closely related to epithelial sodium channels and happens to be selective for small cations. ASIC1a and ASIC3 have been reported in chondrocytes thus far. True to their name, these channels open in response to protons extracellularly. This information matches chondrocyte biology as these

cells are routinely exposed to low pH conditions. Function of these channels has been linked with exposure to low extracellular pH where these channels mediate increase in intracellular calcium concentration, probably required for proliferation and enzyme production.

Connexin 43 hemichannel was found to be active in about 40% chondrocytes constitutively. Possible mode of action of this channel may be that it gets activated by mechanical stimulation and then mediates the release of ATP which will finally increase intracellular calcium by acting via P2 purinergic receptors.

Potassium Ion Channel Super-family

These channels are membrane proteins belonging to one of the most diverse group of channel proteins present in the human genome. There are over 90 reported genes which are known to code for the principal sub unit of these channels. The proposed role of this super family of channels therefore is also quite wide and includes:

a. Stabilizing resting membrane potential
b. Modulation of neuro transmitter release
c. Secretion of hormones
d. Electrolyte transport across epithelium
e. Cellular proliferation
f. Cell volume regulation
g. Involvement in apoptosis

h. Tumor progression

i. Maintenance of potassium homeostasis.

Channel Structure

The tertiary structure of the channel consists of principal (α) and the auxillary (β) sub- units. α sub units determine the structure of the channel since these form the central pore forming ring, whereas the β sub unit (1-4 accessory or auxillary proteins) modifies the properties of the channel. Each α sub unit contains what is known as a 'pore loop' (P domain). This is special because each pore loop lines a specific part of the pore called as the 'selectivity filter'. This is the part of the pore which enables the channel to be selective to the permeant ion. Since there is alternate splicing (genes) and presence of both homomeric and hetero-tetrameric assembly of these principal sub units, there are diverse groups formed within the main family. Each sub unit also boasts of trans-membrane (TM) domains whose number varies considerably and forms the basis for classifying the family into specific groups.

Classification based on number of Trans-membrane (TM) domains

A. 6 TM/ 7 TM domains: each sub unit contains 6 or 7 (in case of large conductance or BK type calcium activated potassium channel) TM domains. 4 such sub units will unite to form members of this group. Eg. Voltage gated potassium channels (K_V) (Figure 2.), Calcium activated

potassium channels (K_{Ca})- large conductance (BK-7TM) (Figure 3.) and small conductance (SK-6TM) (Figure 4.) (40)

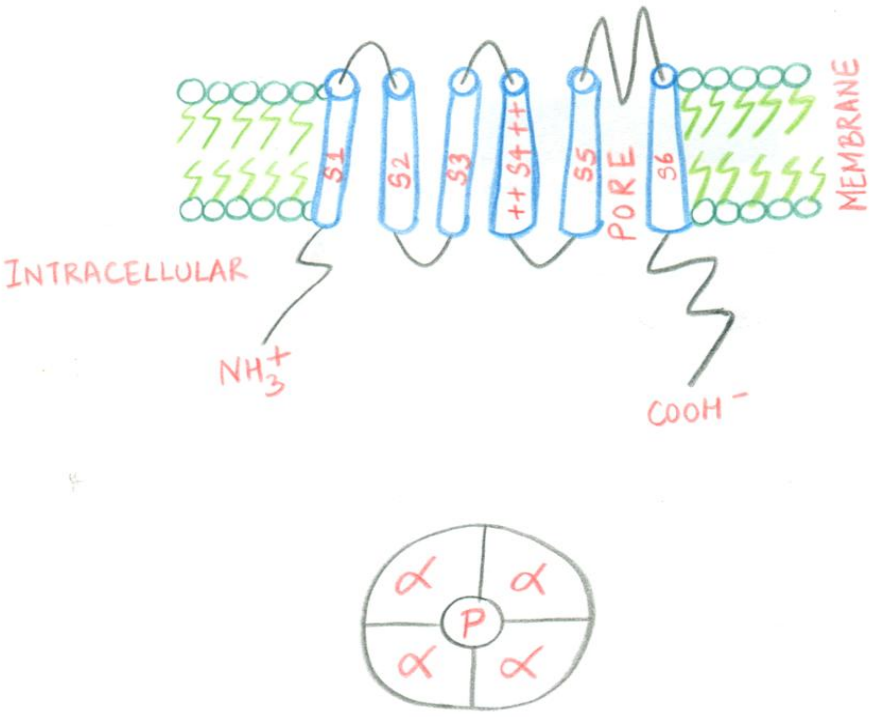

Figure 2. TM topology of Voltage activated potassium channel (K_V) channel: S1-S4 form voltage sensor, S5 & S6 form pore region, Below: Tetrameric arrangement of 4 principal sub units give rise to a functional channel unit.

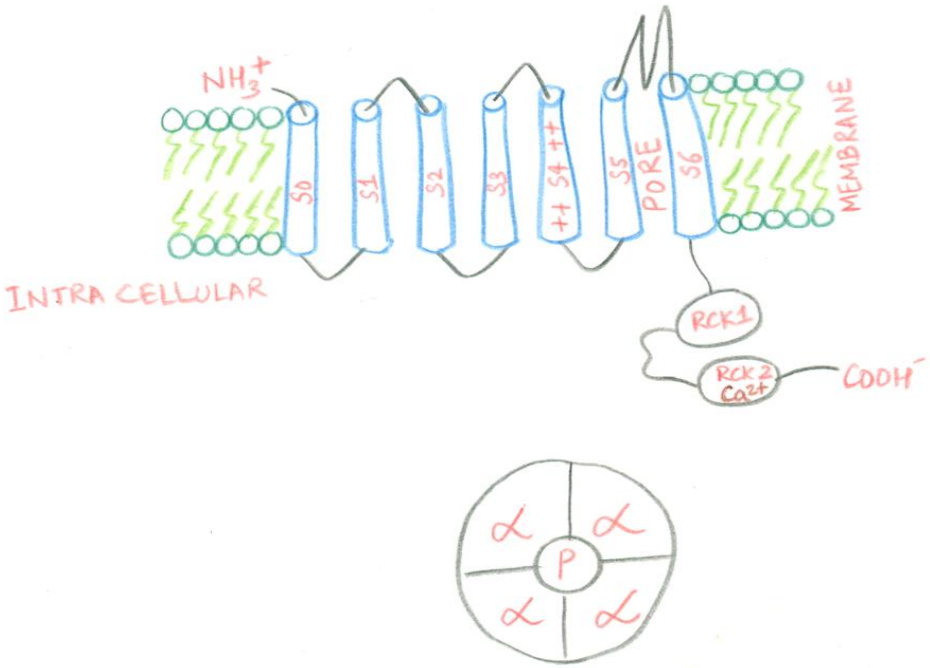

Figure 3. TM topography of BK (big conductance calcium activated potassium channel- K_{Ca}) channel showing 7 TM domains, S1-S4: form voltage sensor with contribution from S0 as well; S5-S6 forming pore region, 2 RCK (regulator of K^+ conductance) domains are in the C terminus with calcium bowl in RCK2. Below: Tetrameric arrangement of 4 principal sub units showing the functional structure of the channel

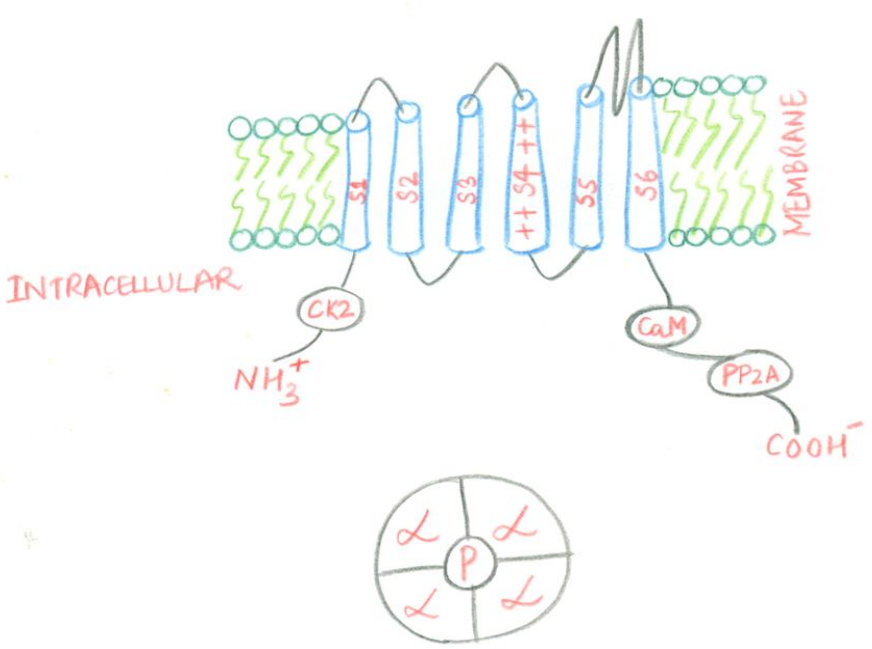

Figure 4. TM topology of SK Channel (small conductance K_{Ca} channel): S1-S4 form the volatge sensor; S5-S6 form pore region, regulators are calmodulin (CaM) and Protein Phosphatase 2A (PP2A) which are seen in the C terminus and Casein Kinase II (CK2) which is present in the N terminus. Below: Tetrameric structure formed by the principal sub units

B. 4 TM domains: the category of channels which come under this group are known as two pore or tandem pore channels since each principal sub unit has two pore domains and the functional structure is probably a dimer. Eg. TWIK, TASK, TALK etc. (Figure 5.) (40)

C. 2 TM domains: This configuration is seen in what are known as inward rectifier potassium channels (K_{ir}) which happen to be a big sub-family of channels in themselves. Since the channel function can get regulated by a lot of other small substances and other interactions, their structure should be studied along with the interacting substance that regulates them. (41) (Figure 6.)

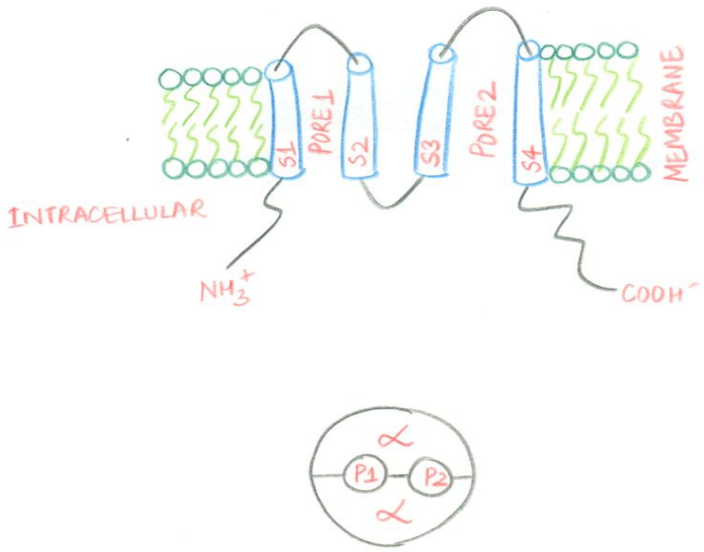

Figure 5. TM topology of two pore channels (K2P channels); S1-S2 form pore region 1 while S3-S4 form pore region 2; Below: Dimeric structure of the channel as assembled by the principal sub units

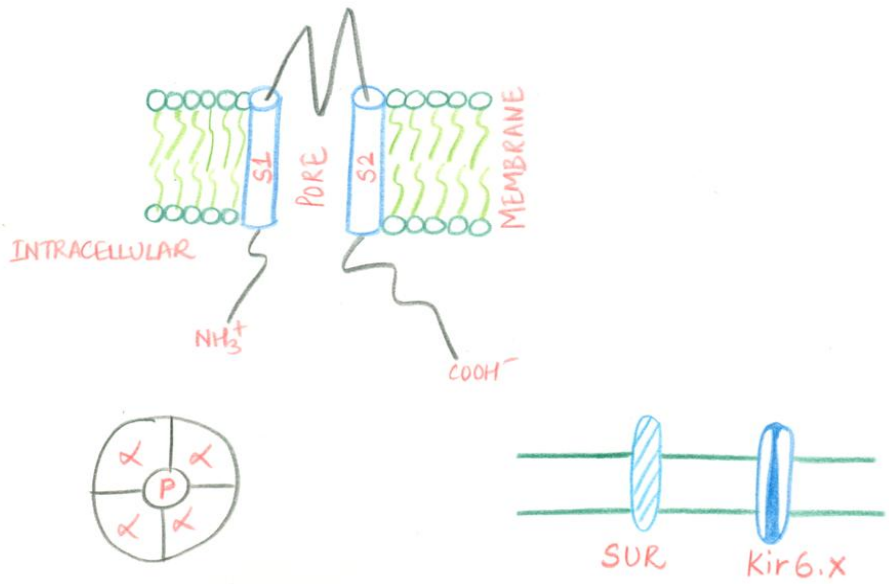

Figure 6. TM topology of inward rectifier potassium channels (K_{ir} channels). S1& S2 form the pore. Below: Tetrameric structure as formed by the principal sub units. Below right: protein-protein interaction regulating channel function eg. Sulfonyl urea receptor (SUR) - K_{ATP}

Voltage gated potassium channels (K_V)

These channels are from the 6 TM domain group and themselves form a big sub family. The principal sub units can be formed of hetero-multimers. The first four TM domains form the voltage sensor while the 4^{th} and 5^{th} TM domains form the linker. The last two domains form the pore region. One of the first to be discovered in chondrocytes, these channels have been reported in multiple studies by various authors. There are reports of these channels being present in

rabbit, caprine, canine and porcine chondrocytes.(37,42–44) The current profile observed in some studies showed a slowly inactivating current now known to be that of delayed rectifier potassium channel. (44) In another study, conducted in our lab, it was observed that the current shows a slow activating and non inactivating profile.(42) There is albeit extensive data to suggest that the potassium channel type present in chondrocyte membrane is mixed and not specific to a particular subtype.(45)

Potassium Channel Modulators & Blockers

There are three categories of modulators for voltage gated potassium channels. They are:

a. Metal ions: eg. Tetraethlyammonium (TEA^+) a quaternary cation used as its salt for channel blockade. The block is dose dependant and effective from both outside and inside the cell. It is one of the most routinely used blockers.(44)
b. Small organic molecules: eg. 4-Aminopyridine (4-AP) This organic compound was first used to demonstrate potassium channel block in cockroach axon and has been in extensive use ever since. (46)
c. Peptides derived from Venom: eg. Charybdotoxin (scorpion venom) has been shown to block the pore region and block the pathway fro ion conduction.(47)

Though ironical but use of these blockers help in the precise identification of these channels and propel further study and analysis.

AIMS

AND

OBJECTIVES

AIM

To compare freshly isolated articular chondrocytes and cryopreserved chondrocytes based on an electrophysiological study.

OBJECTIVES

a. Assessment of cell retrieval and viability following a specific period of cryopreservation using a standardized protocol

b. Comparison of freshly isolated chondrocytes with chondrocytes retrieved after cryopreservation by recording ionic currents using Patch clamp technique.

MATERIALS AND METHODS

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This experiment is divided into the following steps:

Step 1: Isolation of Goat Articular Chondrocytes and their Cryopreservation

Step 2: Performing Patch-Clamp Experiments on Freshly Isolated Chnondrocytes and Cells Procured after Thawing Cryopreserved Chondrocytes.

Step 1: Isolation of Goat Articular Chondrocytes and their Cryopreservation

MATERIALS REQUIRED:

1. Dulbecco's Modified Eagle's Medium (DMEM): bottle containing Nutrient mixture F-12 Ham powder with L-glutamine, 15mM HEPES. It was bought from Sigma (Life Sciences) Stored at 2-8°C till preparation and used at a concentration of 15.6 grams/Litre.
2. Collagenase Type II: Enzyme used for digestion. Purchased from Worthington Biochemical Corporation. (Total quantity- 5 grams, each mg containing 290 units). Stored at 2-8°C. For use- bottle removed and thawed and appropriate quantity (1.5 mg/ml of medium used) was weighed out.
3. Fetal Bovine Serum (FBS): Type- certifies, performance plus, purchased from Gibco(Life Technologies). Quantity- 500 ml which was aliquoted into 14 ml fractions and stored at -20°C.

4. Dimethyl Sulfoxide (DMSO): 500 ml- 1 bottle purchased from Sigma Aldrich. Stored at room temperature, away from direct sunlight. Quantity to be used was syringe filtered before every experiment.

Preparation of Medium:

Requirements:

1. 1 L Sterile De-ionized water
2. 1.2 grams $NaHCO_3$
3. Sterile Penstrep (antibiotic) – 10 ml
4. Amphotericin B – 8 ml
5. 1M NaOH – sterile (to balance pH)
6. Ascorbic Acid – 1 ml
7. 1 L beaker – sterile
8. 1 L measuring Jar – sterile
9. pH Meter
10. Vacuum pump with rubber stop cork
11. Sterile filter

Procedure

For the first step, 200-300 ml sterile De-ionized water was added to the beaker. To this 1.2 g $NaHCO_3$ was added. The solution was mixed between the beaker

and the measuring jar. Next DMEM powder was added. Mixing was repeated again. The solution was made upto 700 ml by adding De-ionized water. pH was adjusted to 7.4 using 1M NaOH solution. 10 ml Penstrep and 8 ml Amphotericin was added next. Finally 1 ml of Ascorbic Acid was added and the volume was made upto 1 L by adding De-ionized water. After thorough mixing, a sterile filter was placed over an autoclaved bottle and screwed tight. The vacuum pump was attached to one end while the other end was sealed using the stop. The lid was removed and medium was gently poured. The pump was switched on and flow was checked. Care was taken to prevent any leaks. Once all medium was filtered, it was aliquoted to smaller bottles to prevent repeated opening and pH change. The bottles were capped tightly and duly labeled (including date). Hood was cleaned and all the waste discarded into appropriate colour coded bins.

Isolation of Articular chondrocytes:

Goat legs were procured from the local abattoir on the day of slaughter. Legs from one animal were considered to be of one set. In this experiment articular cartilage from the metatarso-phalyngeal joint was harvested. Legs from four male goats were used for this study. Chondrocytes isolated from four sets of cartilage were put for cryopreservation and further assessment was done. Legs were either used immediately or refrigerated if to be used, after a few hours. Legs were washed thoroughly, then de-skinned and washed again. They were then

wrapped in tissue paper soaked in 70% ethanol and left for 20 minutes to rid them of contaminants.

DMEM to be used for the procedure was taken out of the refrigerator and thawed before use. Type II Collagenase was also thawed before being weighed. 1.5 mg/ml DMEM used was weighed out and stored in a microcentrifuge tube for further use.

The working surface in the laminar flow hood was decontaminated using 70% ethanol and the legs were taken inside.

Using a sterile blade, all the tendinous attachments were released to expose the joint cavity. A 15 ml centrifuge tube was filled with DMEM to receive the cartilage shavings. Using a new blade, small shavings of cartilage were taken from the joint surface and put into the centrifuge tube. Care was taken not to scrape too deep on the articular surface so as to avoid bone fragments and blood. After shavings from one leg were taken, it was kept aside and similar steps were used for all the other legs. Shavings from two legs were put into one 15 ml tube.

Next a T25 flask was taken and the shavings were transferred to it. Medium was discarded. 1.5 ml medium was added to Collagenase type II (15 mg for 10 ml DMEM used) to dissolve it. This was filled in 10 ml syringe containing 8.5 ml fresh DMEM. Using a fresh syringe filter the contents were transferred to the T25 flask.

The flask was closed and labeled with the Set Number, Date, purpose of use, and transferred to the incubator (37° C, 5% CO_2) for digestion over 16 hours. It was made sure that the flask was left standing in the incubator otherwise all the released cells would adhere to the treated surface. (collagenase II was added in the evening so that digestion could be arrested in the morning to keep the time of enzymatic digestion almost constant)

After 16 hours of digestion were complete, the flask was taken into the hood and the contents were transferred to a 50 ml centrifuge tube through a 40 micron strainer. To arrest digestion equal volume of fresh DMEM was added to the 50 ml tube. After addition was complete, strainer was removed. Contents of the tube were gently triturated. The tube was then capped and centrifuged for 10 minutes at 2500 revolutions per minute (RPM). The supernatant was discarded taking care not to disturb the cell pellet. Fresh DMEM was added to wash the cells which were gently triturated. Centrifugation was repeated again. Supernatant was discarded again and a second wash was given to get rid of enzyme residue and cellular debris. Centrifugation was repeated again and supernatant discarded. The cell pellet obtained was resuspended in 1 ml fresh DMEM. (Figure 7)

CELL COUNT AND VIABILITY

10 microlitre was taken from this cell suspension in a microcentrifugation tube. To this 10 microlitre of Trypan Blue dye (0.4% - GIBCO, membrane filtered, stored at 15-30° C) was added. After thorough trituration, 10 microlitre was taken and cell count was done by charging the Improved Neubauer's Counting chamber and counting the cells in all the 9 big squares. The formula used was:

$$\frac{n}{0.9} \times 2 \times 1000 \times ml$$

Where, n was total no. cells counted in the 9 squares

0.9 was the volume

2000 was the dilution factor

ml signified the total volume of cell suspension

Cell viability was assessed by counting a total of 100 cells and then looking for how many of those cells were viable and how many were dead. The results were tabulated with the cell count for that set. Since 100 cells were counted, we directly got percentage viability for the suspension.

CRYOPRESERVATION

Cryopreservation protocol followed consisted of 3 major steps:

1. Preparation of Freeze medium containing basal medium (DMEM) supplemented with Fetal Bovine Serum (FBS) and addition of Cryoproctective Agent (CPA) to it. Addition of freeze medium to cell suspension and preparing suitable aliquots.
2. Gradual cooling of aliquots containing cells over 24 hours
3. Transfer of aliquots to Liquid Nitrogen Tank for storage

Step 1

Fresh chondrocytes that were to be used for patch clamp analysis were removed from the suspension. The rest of the cells would be used for cryopreservation. The total solution of freeze medium to be made depended on the number of aliquots to be made.(eg. 10 aliquots – total solution prepared 10 ml)

To a 15 ml centrifuge tube, 1 ml cell suspension was added. To this cooled DMEM was added. FBS was added to this diluted suspension to give a final concentration of 20%. This diluted suspension (5 ml) was then stored in ice until further use. In another tube DMEM, FBS and Dimethyl sulfoxide (DMSO) were added to get final concentration of DMEM + 20% FBS + 20% DMSO. DMSO was the cryoproctective agent used in our experiment which helped in vitrification. (Since DMSO releases heat when added to medium, it was added extremely slowly.) The second tube was also stored in ice to keep it cool. While

the suspension and freeze medium were being cooled, cryovials or micro-centrifugation tubes were taken and labeled with the Set Number, no. of cells/ml and date. They were then placed in a holder and kept in the freezer to cool.

The first tube containing 5 ml of cooled dilute cell suspension was taken into the hood and to this freeze medium was added drop-wise. After addition of every drop, the suspension was gently triturated. This was done to avoid osmotic shock to cells. To allow equilibration, time was noted as soon as DMSO containing medium was being added to the suspension. Slow addition and another five minutes of extra time yielded about fifteen minutes for this equilibration to occur.

After addition of 5 ml of freeze medium, the cell suspension had a final concentration of DMEM+ 20% FBS + 10% DMSO (total 10 ml). The holder containing the cryovials was removed from the refrigerator and to each cryovial 1 ml of suspension was added after thorough but gentle trituration.

Step 2

After all the vials were filled, they were quickly transferred to a 1° Thermo cooler (Cryo 1° C cooler- bought from Tarsons Products. The cooling rate achieved by this container when filled with a coolant like isopropyl alcohol is approximately 1° C per minute). The thermo cooler was already filled with Isopropyl alcohol and pre cooled to -20° C before transferring the cryovials. This

thermo cooler was then kept in -80° C Freezer for 24 hours for gradual cooling of cells over time.

Step 3

After 24 hours the thermo cooler was taken out of the Freezer and the cryovials were quickly transferred to a cylinder carrying Liquid Nitrogen. The cylinder had removable containers into which cyrovial holders could be placed and removed with minimal handling. Care was taken that cryovials did not thaw while they were being transferred to the cylinder.

Level of liquid nitrogen in the tank was checked and recorded two times every week. About 4 litres of Liquid nitrogen was added to the tank every week to prevent thawing of cells. (Liquid nitrogen keeps evaporating from the tank and therefore level needs to be checked periodically)

CELL RETRIEVAL & VIABILITY

For assessment of retrieval and viability following cryopreservation, chondrocytes were divided into two groups based on duration of cryopreservation:

1. Group 1: Day 7 to Day 10 of cryopreservation
2. Group 2: Day 11 to Day 15 of cryopreservation

On Day 7 of cryopreservation, 1 Aliquot was carefully removed from the Liquid Nitrogen Tank making sure that other aliquots were not disturbed. Once out, the aliquot was checked to make sure that the suspension was in frozen state within the tank and had not thawed (Figure 8). Once out, the cryovial was thawed in a 37°C water bath or gently rolled within the palms of the hands. Meanwhile DMEM was taken out of the refrigerator and made to thaw. The cryovial and Medium bottle were then taken into the laminar flow hood. Suspension from the cryovial was transferred to a 15 ml centrifuge tube. Fresh DMEM was then added to this cell suspension (five times the volume of suspension) and gentle trituration was done. Five minutes were given for the DMSO to come out of the cells and the suspension to stabilize. The tube was then centrifuged for ten minutes at 2500 RPM. Supernatant was discarded and the cell pellet was washed with fresh DMEM followed by gentle trituration. Centrifugation was repeated for 10 minutes at 2500 RPM. A second wash was given and a final centrifugation step was done. The supernatant was discarded and the cell pellet was resuspended in 1 ml fresh DMEM. 10 microlitres was taken from the suspension to perform cell count for retrieval and viability (Figure 7.).

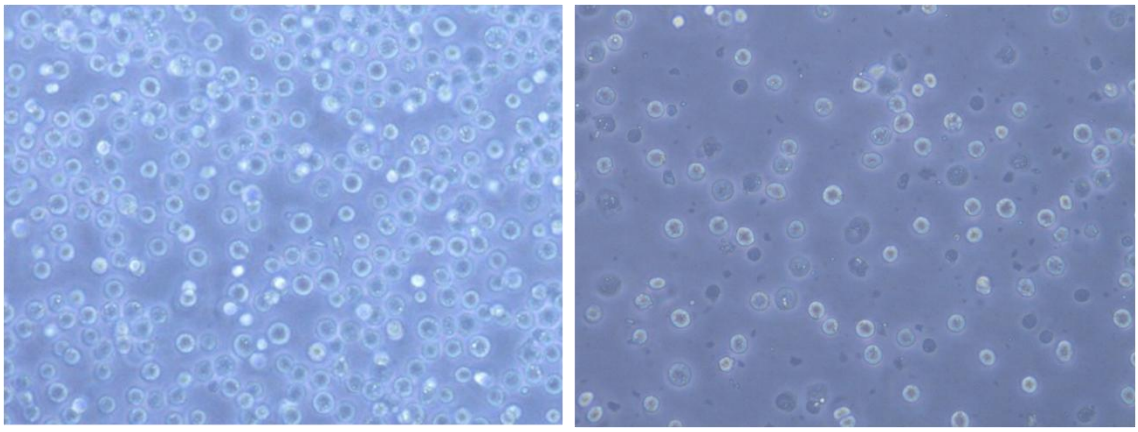


Figure 7. Panel shows microscopic appearance of freshly isolated chondrocytes (Left) and Cells retrieved from cryopreservation on Day 8 (Right). (Suspension prepared in DMEM- 200X)

Figure 8. Tube on retrieval from Liquid Nitrogen Container shows cell suspension in frozen form

Cell Count

To the 10 microlitres of cell suspension, an equal volume of Trypan Blue dye was added. After careful mixing, 10 microlitre was withdrawn from this mixture and the Improved Neubauer's chamber was charged. Cell count was done by counting cells in all the 9 big squares as described above. The total number of cells counted was the amount retrieved from that aliquot and was recorded as such. Percentage viability was also determined by counting 100 cells and the values were recorded separately (Figure 9). The same protocol was followed for cells retrieved on Day 8 through 15 and the information recorded was divided into the two arms as mentioned before.

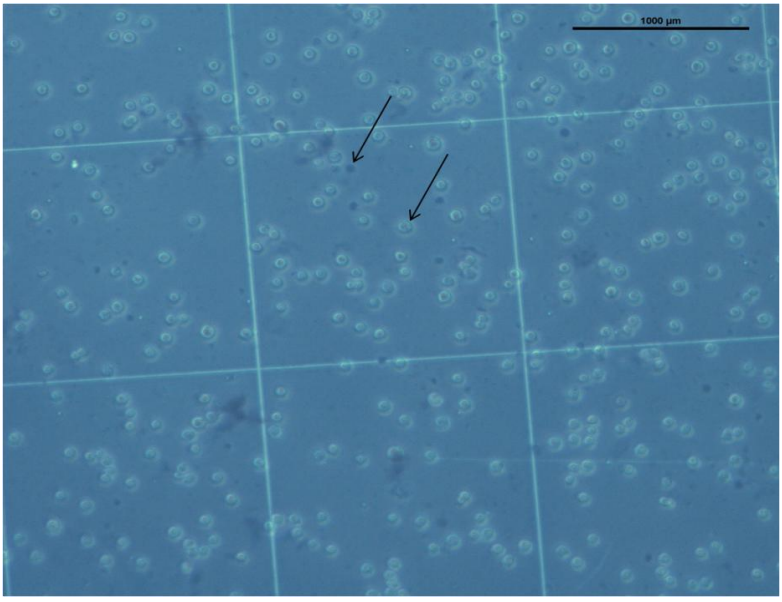


Figure 9. Cells retrieved on Day 8 of cryopreservation (100X) - Stained with Trypan blue dye to check viability. Viable cell are seen as refractile bodies whereas dead cells are completely stained.

Step 2: Performing Patch-clamp experiments on freshly isolated chnondrocytes and cells procured after thawing cryopreserved chondrocytes.

MATERIALS REQUIRED:

1. Freshly isolated chondrocytes (Day 0)
2. Post thaw cryopreserved chondrocytes from both Group 1 & Group 2
3. Bath Solution (corresponding to ECF solution)
4. Pipette Solution (corresponding to ICF solution)
5. 10 mM TEA^+ in Bath solution

   (Tetraethylammonium chloride- TEA-Cl: 25 grams, bought from SIGMA. 1M stock solution prepared and refrigerated for later use.)
6. 35 mm Treated Petridishes
7. Glass Micropipettes
8. Sylgard
9. Disposable syringes
10. Microcentrifige Tubes
11. Pipette and tips
12. Patch clamp set up (described later)

Preparation of Bath and Pipette Solution

Both the solutions were prepared in autoclaved De-ionized water. To half the volume of de-ionized water, salts were weighed according to final concentration desired and added. pH was checked using pH meter and adjustments made using 1M NaOH. After addition of all salts, water was added to make up final volume. (Table containing composition given after description- Table 1 and Table 2) While making bulk solution, Glucose was not added. Instead stock of 1M glucose was prepared separately and stored at -20°C. While performing an experiment, a small quantity was reconstituted with glucose and used. The prepared solutions were stored in the refrigerator unless used.

Preparation of 10 mM TEA^+ in Bath solution

A 1 M stock solution of TEA was prepared and refrigerated. For use in experiments, one part from stock was diluted 100 times using bath solution and final concentration was achieved.

Table 1: BATH SOLUTION COMPOSITION

NaCl	140 mM
KCl	5 mM
$MgCl_2$	1 mM
$CaCl_2$	1 mM
HEPES	10 mM
Glucose	10 mM

pH titrated to 7.4 (using 1M NaOH)

Table 2: PIPETTE SOLUTION COMPOSITION

KCl	140 mM
$MgCl_2$	2 mM
HEPES	10 mM
Glucose	10 mM

pH titrated to 7.35 (using 1M KOH)

Osmolarity was checked before performing experiments and range of values was:

Bath solution: 290-310 mOsm/L

Pipette solution: 280-290 mOsm/L

Preparation of cells for patch clamp analysis

After completing cell count and testing for viability, cells suspended in fresh DMEM were plated onto treated 35 mm individual petridishes. Approximately 50,000 cells were plated per petridish which was then placed into the CO_2 incubator at 37°C for fifteen minutes. This allowed the cells sufficient time to adhere to the bottom of the dish so that during patching they would not drift away. After about 15 minutes additional 2 ml medium was added to each dish and they were again placed into the incubator until required.

Micropipette fabrication

Borosilicate patch clamp quality glass tubes (Kimax-51 melting point capillary tubes) were used to prepare the micropipettes. This tube had the following dimensions:

- outer diameter 1.8 mm
- inner diameter 1.5 mm

Capillary tube ends were initially flame polished so as to make them smooth for introduction into the pipette holder later. To pull the pipettes, a two step gravity assisted Narishige Model PP-830 pipette puller was used. The temperature setting on the pipette puller was standardized to furnish pipettes with the resistance of the order of 1.2-2.8 mega ohms (when filled with pipette solution). They were set at: for first step temperature of the filament rose to 60.4°C and for the second step it was fixed at 49.6°C the pipette tips were then polished under

microscopic guidance using GlasswoRX F-500 Fine point microforge with the filament diameter being 0.002 inches. The polished micropipette tips were then coated with Sylgard, which is a silicone elastomer (known to solidify on exposure to high temperature) so as to decrease pipette capacitance while recording. Care was taken not to block the mouth of the tip while using Sylgard therefore it was done under optimal lighting conditions. Following application of Sylgard, tip was held in front of a hot air blower to set it.

Fresh micropipettes were prepared on each day when an experiment was planned and were used within 3-4 hours of fabrication. They were stored in clean boxes which were kept closed to prevent any dust accumulation.

Cell mounting under the microscope

Petridish was recovered from the incubator and the medium was gently removed. 2 ml bath solution was then added to the petridish. Volume of bath solution added was kept constant for all experiments. Experiments were performed within a Faraday cage fixed on a table designed to isolate vibration. The cage helped reduce noise while recording by isolation of the entire setup from electro-magnetic waves. A chloride coated silver pellet served as the bath electrode which was immersed into the bath solution so as to complete the electrical circuit. The micropipette when held by the pipette holder enclosed within it a fine silver wire coated with silver chloride to establish electrical connection. Only healthy looking cells with intact membrane were selected for patch clamp

recording. This was done by observation aided by the microscope under high power. This step proved important as cryopreservation yielded a suspension having a significant number of dead cells. Healthy cells appeared refractile and a distinct cell membrane could be easily made out. While non viable cells looked dark and sometimes membrane disruption was evident. Another consideration was to choose cells which had firmly attached to the bottom of the dish so that there was no drift of the cell when it was touched by the pipette. Using tip-dip and backfill, pipette solution was filled into the micropipette and all air bubbles eliminated by gentle flicking. (Air bubbles may block pipette tip resulting in increased resistance) pipette was then fixed into the pipette holder and lowered into the bath slowly till the tip just touched the bath solution. Resistance was recorded and pipette capacitance was cancelled.

Making a recording

Pipette tip was brought under focus and slowly lowered over the cell till it just touched the membrane. Contact was confirmed by slight increase in the resistance. Negative pressure in the form of suction via a syringe was then applied to the pipette to attain a seal between the pipette and the membrane which was signified by increase in resistance to Giga ohm. (Giga-seal) Holding voltage was changed to -80 mV. This was followed by sharp suction to disrupt the membrane and break into the cell. Successful access was confirmed by appearance of cell capacitance transient. Before cancellation, cell capacitance

was measured using the Membrane test function provided by Clampex 9.2 software. This provided information about cell capacitance, membrane resistance, access resistance and time constant. Using the whole cell parameters, cell capacitance was cancelled before proceeding with the actual current recordings. Different protocols were used to make specific recordings which have been described below.

All the data was acquired using Axopatch 200B patch clamp amplifier. Digitization was done using Axon Instruments Digidata 1322A analogue-digital converter. For online filtering of data, a 10 kHz low pass Bessel filter was used. To reducing unwanted signals and noise, offline filtering was done during analysis wherever required. The sampling rate used while making recording using the set up was fixed at 50 kHz. pClamp software was used for acquisition and partial analysis of data. Data was also exported to Microsoft Excel and IgorPro software used to generate graphs and proceed with further analysis. (Details with each protocol)

Protocol Design

Protocol used for recording voltage gated Potassium channel currents:

The membrane was held at -80mV (milivolts) during rest. This was the holding voltage or V_{Hold}. Thereafter the membrane potential was clamped from -80 mV to +70 mV with an increment of 10 mV for each step or each sweep giving a total of sixteen sweeps. This was the test pulse used to make the recording. These

depolarizing steps were used to open voltage gated potassium channels and record currents originating from them. The duration of each pulse was set at 200 mili seconds. After each sweep the membrane was brought back to the holding potential of -80 mV. Inter-pulse interval was fixed at 5 seconds. (Figure 10)

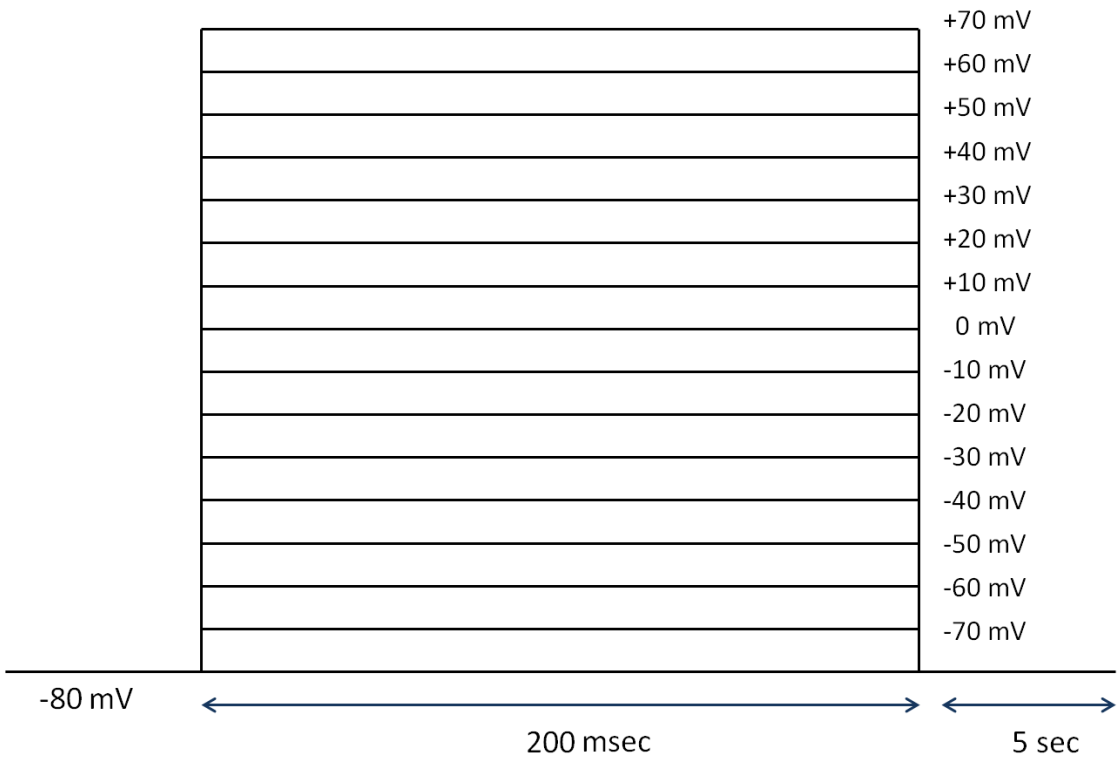


Figure10. Protocol used for recording K_V currents. V_{Hold}: -80 mV, depolarizing steps ranging from -80 mV to +70 mV with an increment of 10 mV

Protocol used for measuring Leak currents

Membrane was held at -80 mV during rest. From this holding voltage, steps of hyperpolarizing potentials were given ranging from -80 mV to -140 mV with a

change of 15 mV. In total there were five sweeps. Each test pulse had duration of 100 msec and after each pulse membrane voltage was brought back to -80 mV. Inter-pulse interval was fixed at 40 msec. Hyperpolarizing pulses were used to measure leak currents (time independent currents) so that they could be studied and subtracted from the current of interest during analysis. (Figure 11)

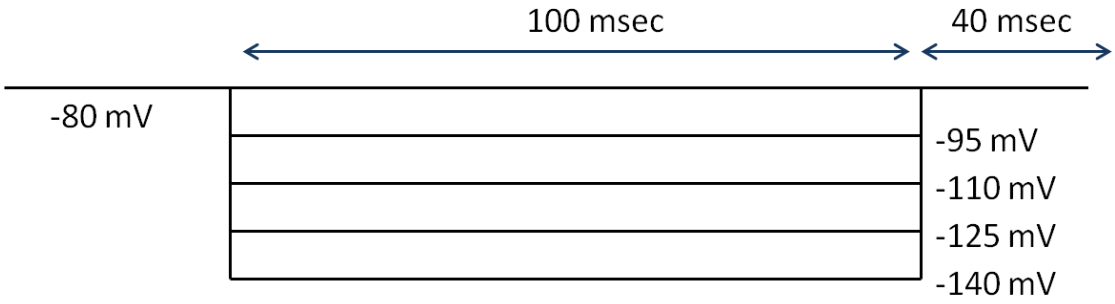


Figure 11. Protocol used to measure leak current. V_{Hold}: -80 mV, hyperpolarizing step voltages ranging from -80 mV to -145 mV given, with change of 15 mV

Protocol for measuring Reversal potential

Membrane was held at -80 mV when at rest. From this holding potential, a depolarizing step was applied and voltage clamped at +60 mV. The duration of this depolarizing pre-pulse was 200 msec. Following this pre-pulse, voltage was clamped at potentials ranging from +20 mV to -140 mV with a change of 20 mV. The total number of sweeps therefore was 9 in number. Duration of each step

was 60 msec following which membrane potential returned to -80mV. Inter-pulse interval was fixed at 4 seconds. The depolarizing pre-pulse was used to open the channel. Test pulses yielded tail currents from which reversal potential was measured (Figure 12). Comparison with calculated Nernst equilibrium potential was made to confirm the permeable ionic species.

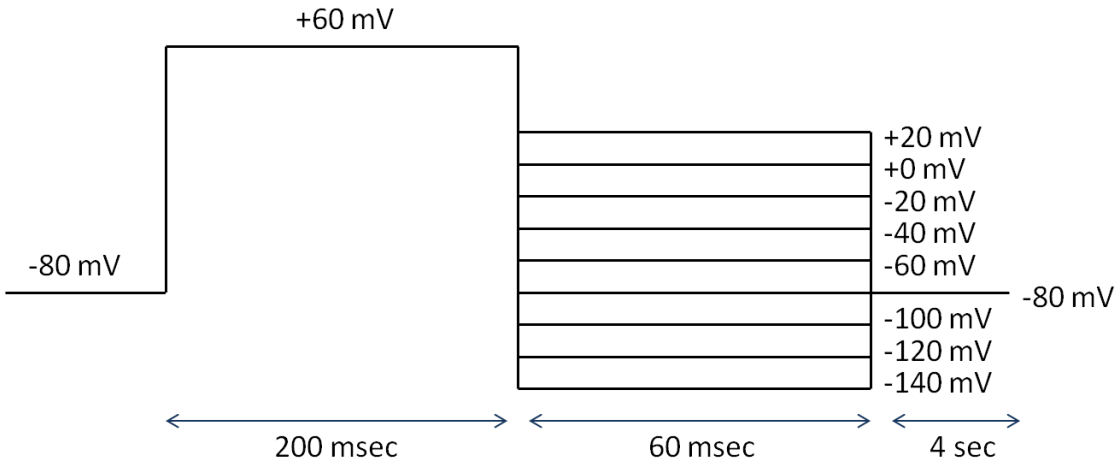


Figure 12. Protocol used to record tail currents: V_{Hold}: -80 mV, followed by depolarizing pre-pulse to +60 mV, and voltage clamped from +20 mV to -140 mV with a change of 20 mV.

Calculation of Nernst Equilibrium Potential (For Potassium)

$$E_{K^+} = -\frac{RT}{zF}\ln\frac{[K^+]_i}{[K^+]_o}$$

Where,

i. E_{K}^{+} is membrane potential (in volts)

ii. R is Ideal gas constant

iii. T is temperature in Kelvin

iv. F is Faraday's constant

v. z is valency of the ion

Effect of Blocker: TEA^+

To study the effect of TEA^+ on the currents obtained, ALA Scientific Instruments' OCTAFLOW II perfusion set up was used. To control the pressure in the valves and outlet selection, Octaflow software was made use of.

Focal perfusion was used to deliver the blocker to the chondrocyte being patched. The gas used to generate pressure in the system was Nitrogen. After standardization, a pressure of 3.5 psi was used to perfuse the cell. After preparing and checking the entire setup, capillary outlet for perfusion was brought into focus and lowered into the bath until it just reached the base of the dish (no

contact). Then it was placed at the periphery of the field so as to carry out manipulation of patch pipette without hindrance. To keep conditions similar for all recordings, bath perfusion at 3.5 psi pressure was used for making standard recordings to which other recordings made with the blocker were compared. 10 mM TEA^+ in bath solution was used as the potassium channel blocker and was focally perfused at 3.5 psi. In case a recording with the blocker had been made, the bath was discarded and a new petridish with fresh cells was used for next recording.

Analysis of Data acquired

Voltage gated Potassium currents

Magnitude of the current at each test potential was obtained after it had reached a steady state level. Currents were averaged using a time duration lasting for about 20 msec. This step was used to reduce the influence of noise. The current magnitudes thus obtained were exported and saved. In a similar manner leak current was also estimated and the values saved. The value of leak current was subtracted from the peak current amplitude obtained from the current tracing for every membrane potential in the protocol. The values thus obtained were divided by the cell capacitance (which had already been measured using the membrane test feature) to yield the current densities at various voltages. Mean and standard

deviation was calculated. The data was exported to IgorPro for further analysis and pictographical representation.

Calculation of Reversal potential

Current-voltage (I-V) curves were generated from the tail current tracings obtained from the protocol used. I-V curves were also generated to obtain sub-threshold leak currents from the same tracing. This leak current was subtracted from the peak current to get leak subtracted current which was plotted to get reversal. This value was compared with calculated reversal potential to identify the permeant ion species.

Effect of extended cryopreservation

Aliquots from one set (set no. 2) were cryopreserved for 30 days and retrieved using the protocol mentioned earlier. Viability and patch clamp analysis was done. Results were tabulated separately as the sample size for this group was small.

Statistical Analysis

Data was analyzed using. SPSS software (17.0 version) was used for final data analysis. Kruskal-Wallis Test (non-parametric) was applied to get the desired values at significant voltages. Independent samples Mann-Whitney U Test was used to determine inter-group statistical significance. A p value of 0.05 was considered as significant.

RESULTS

RESULTS

COMPARISON OF VIABILITY

FRESH ISOLATED CHONDROCYTES:

Cell viability in freshly isolated chondrocytes from cartilage shavings obtained from all four sets was found to be 99% (Figure 13).

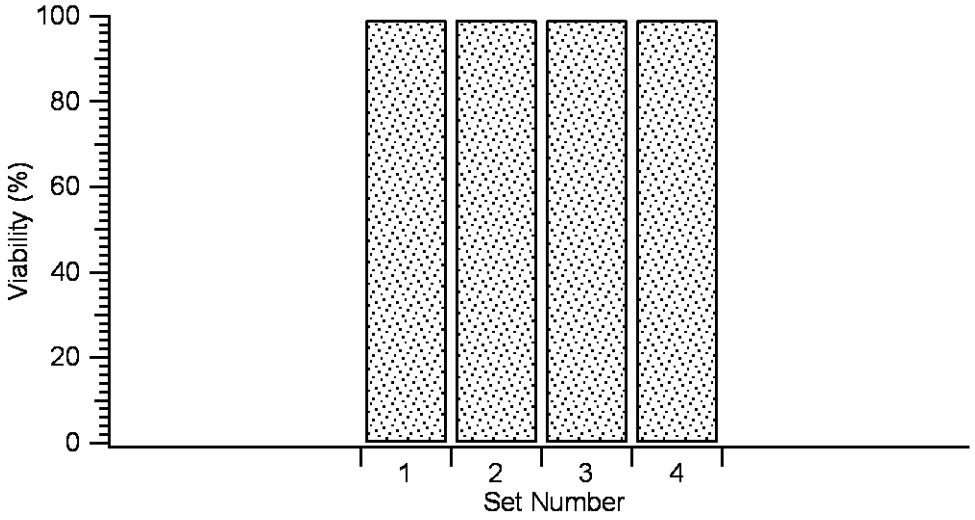


Figure 13. Cell viability across sets in freshly isolated chondrocytes

RESULTS OF CRYPORESERVATION:

SET 1: The number of viable cells retrieved per million cells cryopreserved was 0.65 million on Day 7 and reduced to 0.08 million on Day 15. Percentage

viability on Day 7 was recorded to be 70% which reduced to 10% by Day 15 (Figure 14).

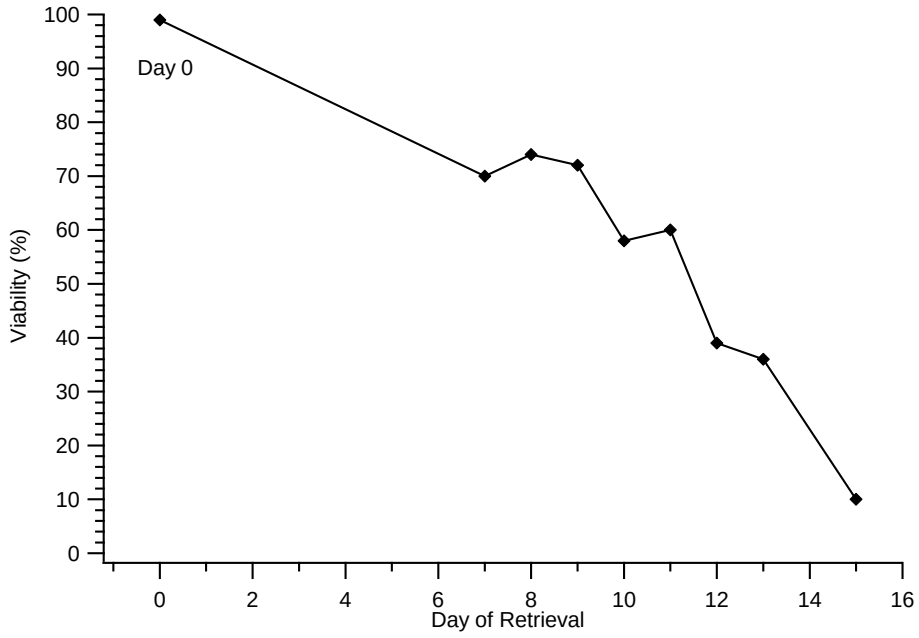


Figure 14. Graph expressing percentage viability over time of retrieval from cryopreservation. Day 0 represents freshly isolated chondrocytes. (Set 1)

SET 2: The number of viable cells retrieved per million cells cryopreserved was 0.5 million on Day 7, increased to 0.7 million on Day 8 and again reduced to 0.59 million on Day 15. Percentage viability on Day 7 was recorded to be 70% which reduced to 66% by Day 15 (Figure 15). 2 aliquots stored for an extended

period (30 days) resulted in a retrieval of 0.4 million cells per million cells cryopreserved. Percentage viability was 58%. (Table 3)

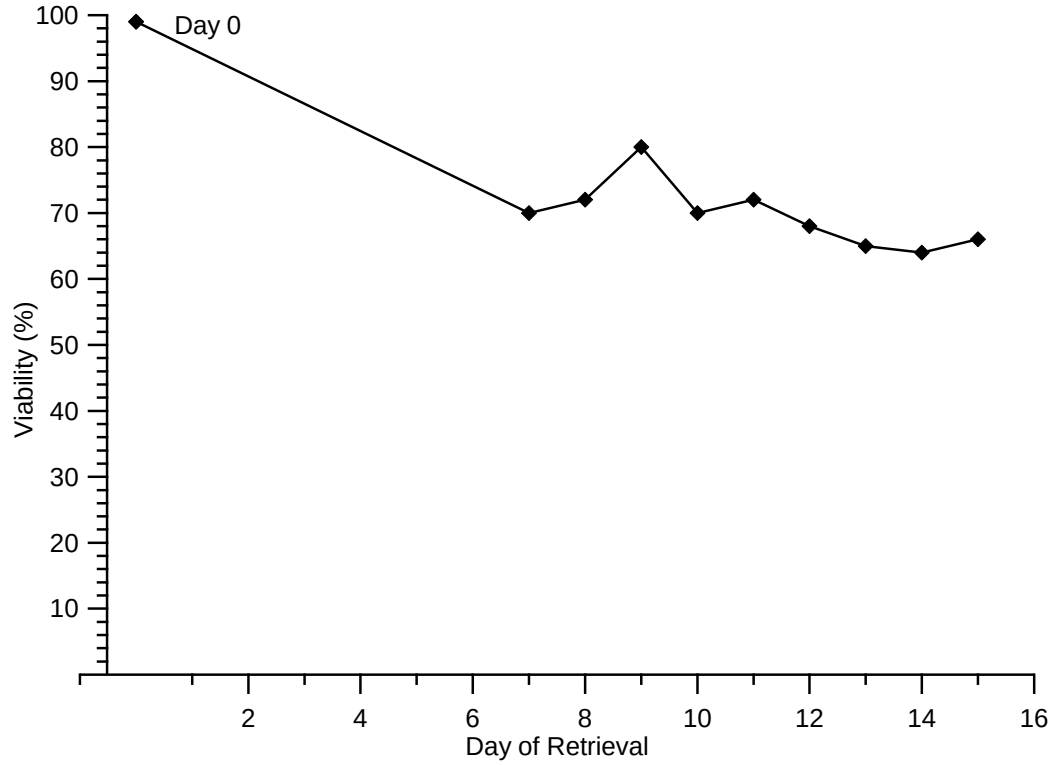


Figure 15. Graph expressing percentage viability over time of retrieval from cryopreservation. Day 0 represents freshly isolated chondrocytes. (Set 2)

SET 3

Chondrocytes put for cryopreservation from set 3 on retrieval did not show any cells therefore count was not possible. Both stained and unstained cell suspension

was checked for viable or dead cells but none were found. Suspension stained with Trypan blue showed cellular debris. Cell suspension aliquots retrieved on all successive days (as specified in protocol) yielded a similar result

(Details of retrieval & % viability for all sets provided later in results-Table 3)

SET 4: The number of viable cells retrieved per million cells cryopreserved was 0.84 million on Day 7 and reduced to 0.61 million on Day 15. Percentage viability on Day 7 was recorded to be 88% which reduced to 68% by Day 15. (Figure 16)

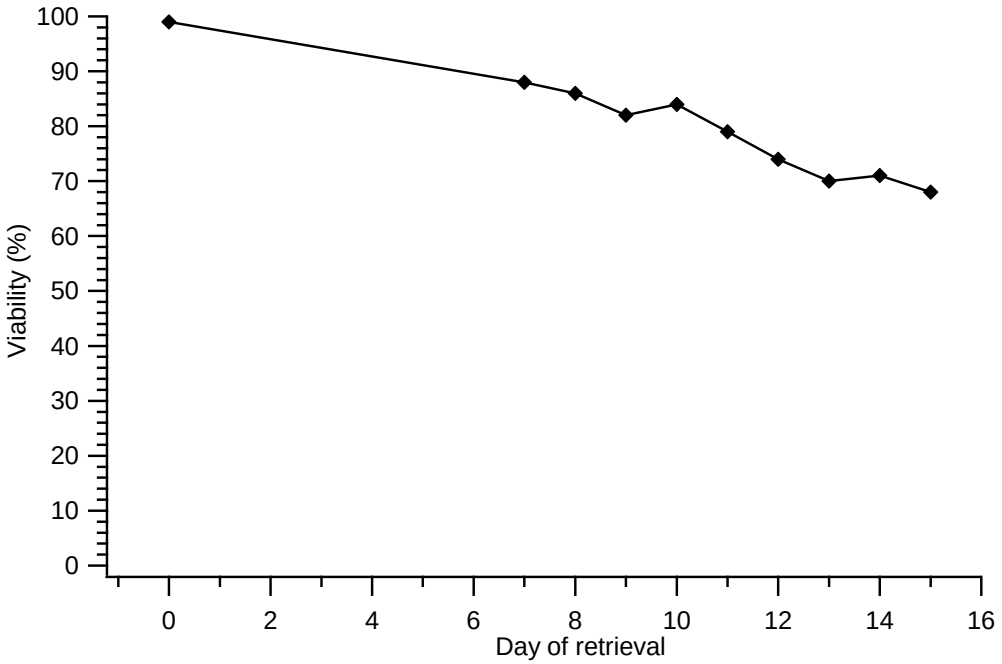


Figure 16. Graph expressing percentage viability over time of retrieval from cryopreservation. Day 0 represents freshly isolated chondrocytes (Set 4)

Table 3. Data representing cell retrieval and percentage viability following cryopreservation of goat articular chondrocytes. Data presented includes values from 4 different sets.

DAY OF RETRIEVAL	No. of viable cells retrieved per million cells cyropreserved (in millions)				VIABILITY (%) Trypan Blue Dye Exclusion			
	SET 1	SET 2	SET 3	SET 4	SET1	SET2	SET3	SET4
Day 7	0.65	0.50	0	0.84	70	70	0	88
Day 8	0.59	0.70	0	0.82	74	72	0	86
Day 9	0.53	0.76	0	0.74	72	80	0	82
Day 10	0.35	0.67	0	0.75	58	70	0	84
Day 11	0.49	0.65	0	0.69	60	72	0	79
Day 12	0.17	0.62	0	0.68	39	68	0	74
Day 13	0.35	0.61	0	0.70	36	65	0	70
Day 14	-	0.60	0	0.68	-	64	0	71
Day 15	0.08	0.59	0	0.61	10	66	0	68
Day 30	-	0.40	-	-	-	58	-	-

PATCH CLAMP: RESULTS

An analysis of currents recorded in freshly isolated chondrocytes using the protocol mentioned in methods section, revealed presence of voltage sensitive outward currents. Magnitude of current varied across cells that were patched. Currents with peak amplitude of about 8300 pA were recorded (Figure 17).

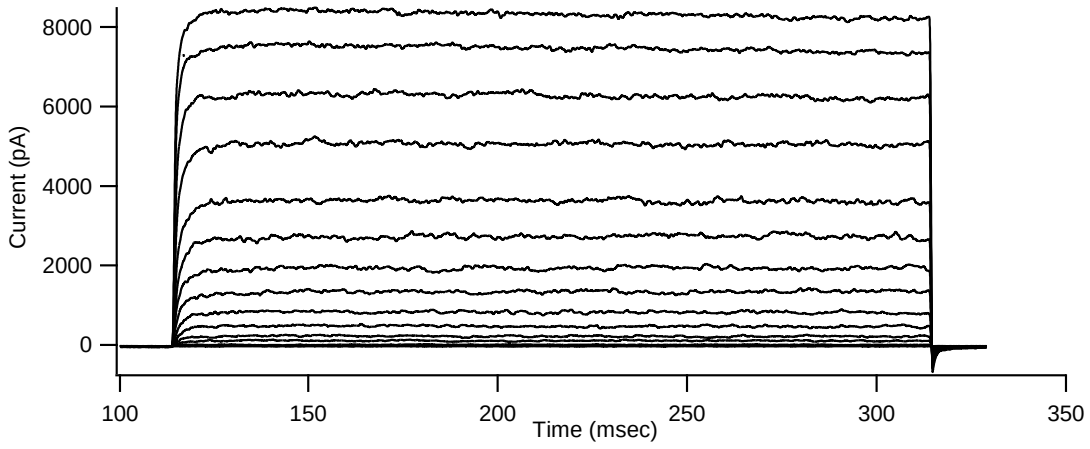


Figure 17. Representative current tracing, as seen in freshly isolated chondrocytes

Current Kinetics

The I-V curves generated from the raw tracings showed the presence of a slowly activating, non- inactivating, outwardly rectifying current. This was suggestive of voltage gated Potassium current (Figure 18 and Figure 19).

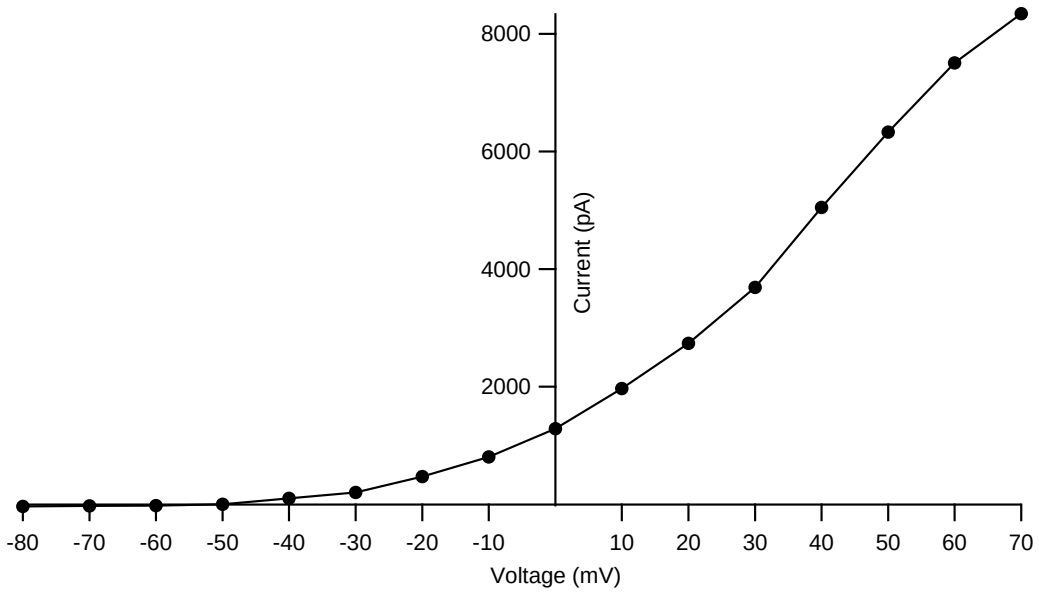


Figure 18. shows representative I-V curve without Leak subtraction as plotted from current tracings obtained from freshly obtained chondrocytes and cryopreserved cells.

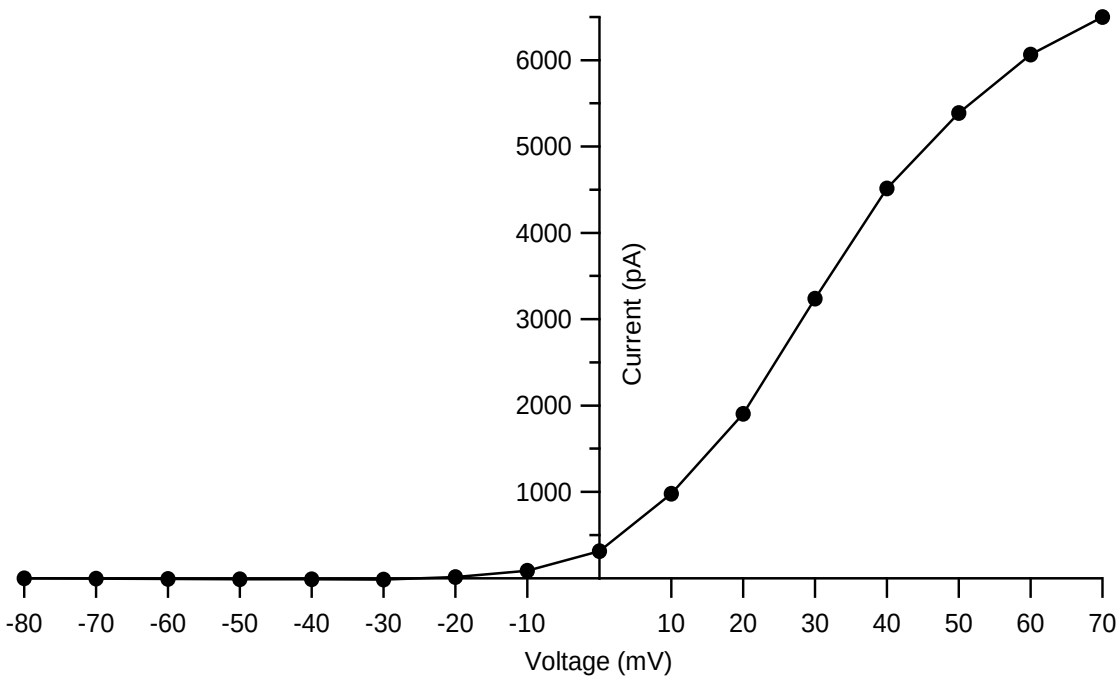
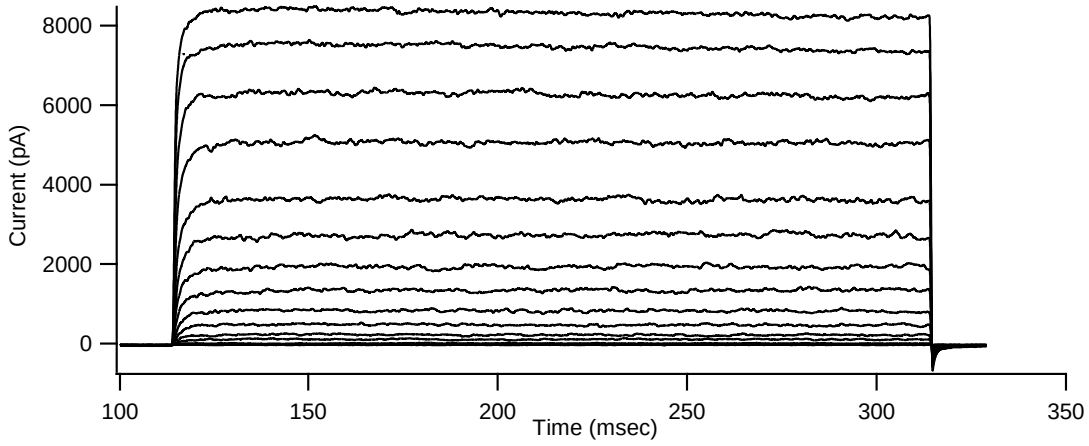


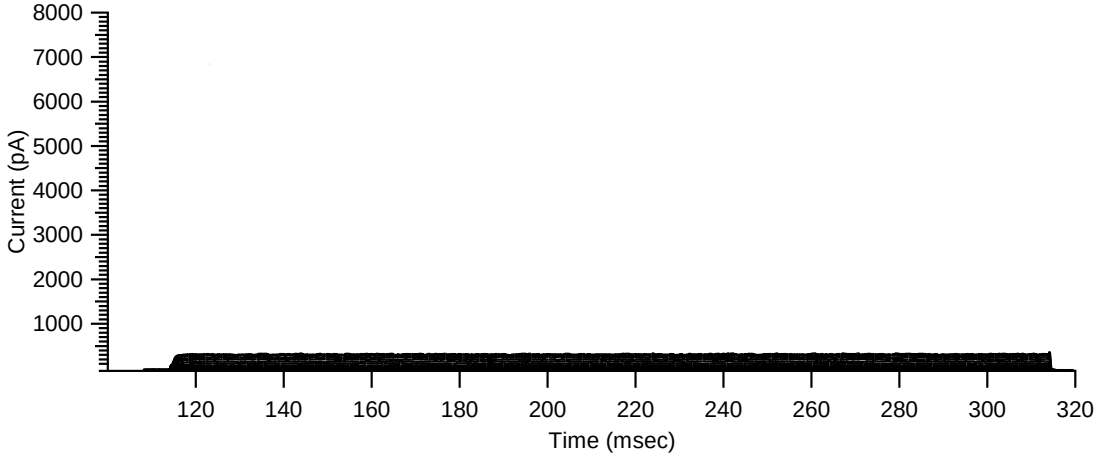
Figure 19. shows representative I-V curve after Leak subtraction as plotted from current tracings obtained from freshly obtained chondrocytes and cryopreserved cells.

Effect of TEA^+ (Potassium channel blocker)

10 mM TEA, used with focal perfusion as blocker caused significant reduction in current amplitude in all cells patched. Since TEA^+ is a known Potassium channel blocker, the current seen here is identified to be of the voltage gated potassium channel sub-family (Figure 20A, 20B and Figure 21)



(A)



(B)

Figure 20. Current tracing obtained from a fresh chondrocyte when depolarizing steps were applied V_{Hold}= -80 mV, Depolarizing pulse ranging from -80 mV to +70 mV was used with an increment of 10 mV (A) when perfused with bath solution at 3.5 psi and (B) when perfused with 10 mM TEA^+ at 3.5 psi

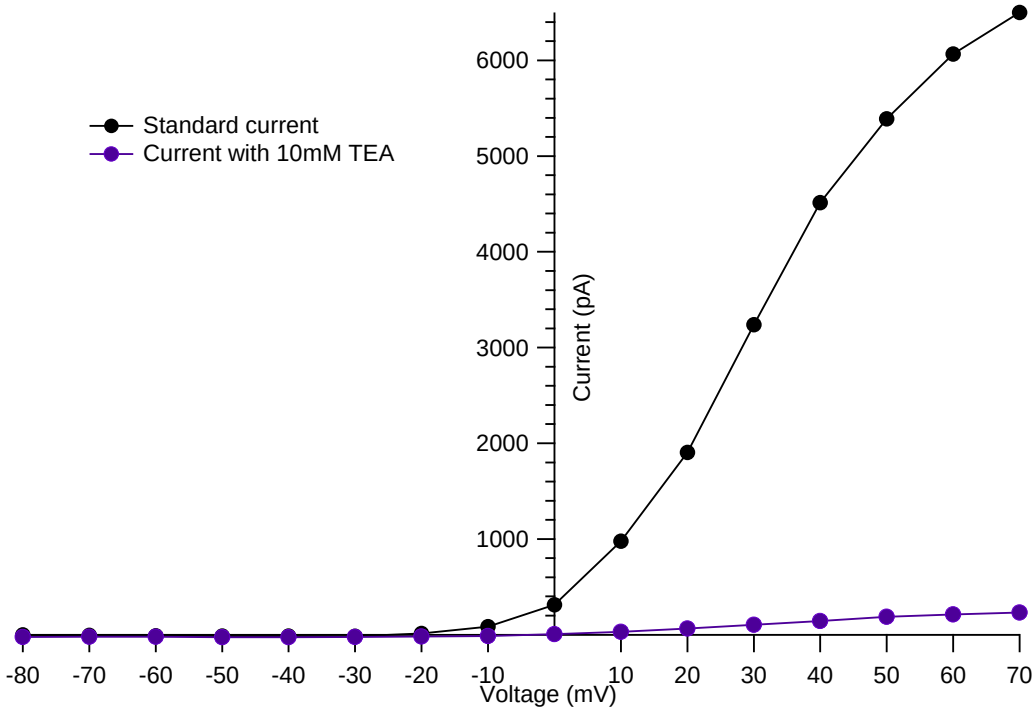


Figure 21. Representative I-V curve before and after addition of 10 mM TEA^+ showing drop in peak current magnitude at every voltage step seen after addition of 10 mM TEA^+

Measurement of Reversal potential

Reversal potential obtained from the instantaneous tail current I-V curves (Figure23.) was seen to be in the range of -75 to -69 mV which was close to the

calculated Nernst Equilibrium potential for potassium. (Calculated value = -84.7 mV) This shows that the permeant ionic species causing the outward current is (in part) Potassium.

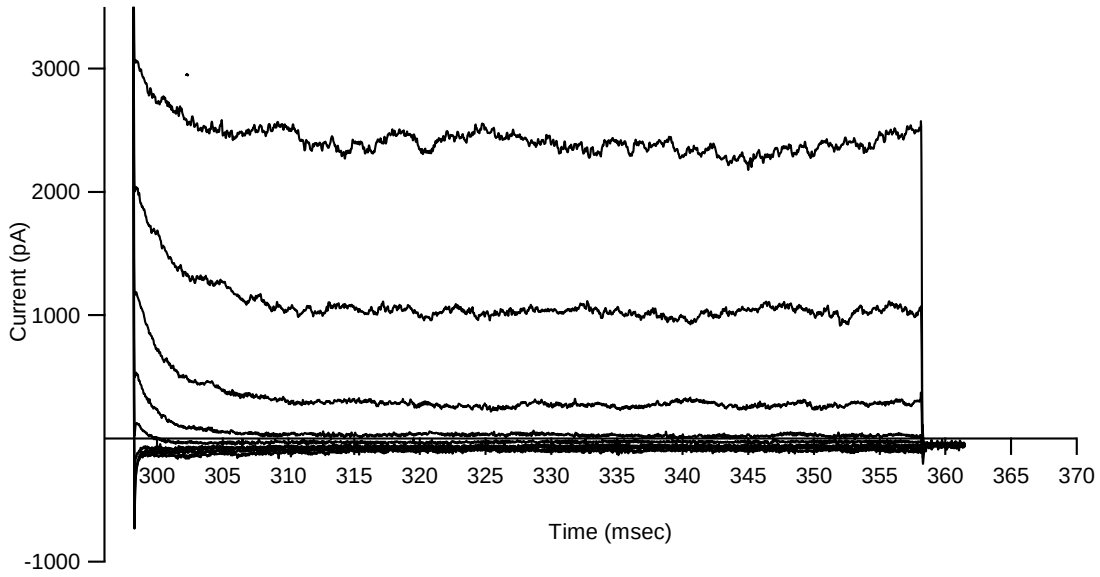


Figure 22.Representative tail current tracing over time in a freshly isolated chondrocyte. Following a depolarizing prepulse to +60 mV, voltage was clamped at potentials ranging from +20 mV to -140 mV with a change of 20 mV. Tracing here portrays the current at those potentials.

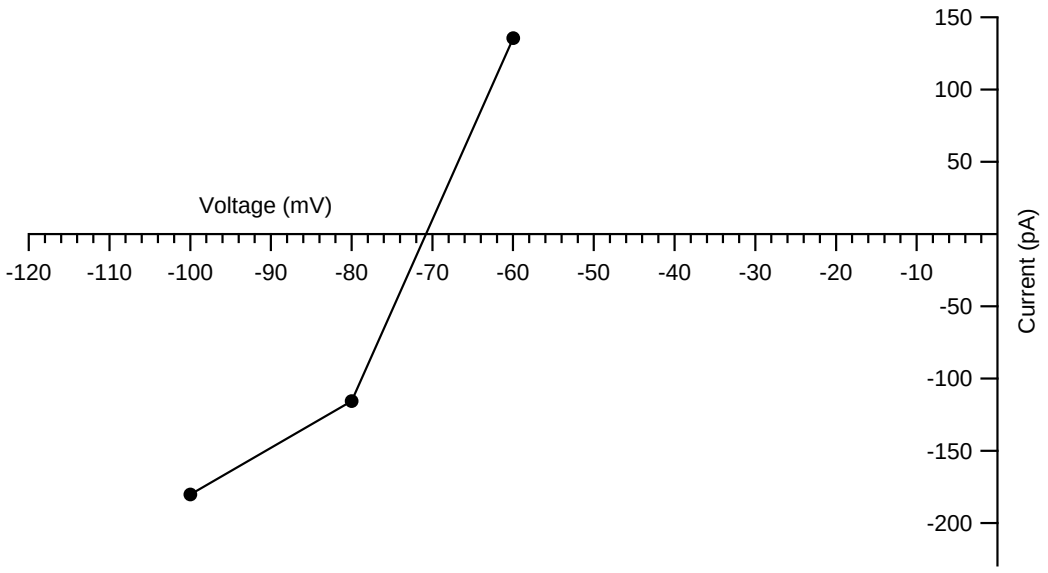


Figure 23. Representative leak subtracted instantaneous tail current I-V curve depicting reversal potential- for a freshly isolated chondrocyte

Inter-Group comparison

A comparison of current tracings revealed similar profile in freshly isolated chondrocytes (n=6) and cryopreserved cells from both groups. This data, showing similar voltage sensitive outward currents even in cryopreserved cells is suggestive of the presence of voltage gated potassium channels. Figures 24, 25 and 26 show change in current density (obtained after leak subtraction and dividing by cell capacitance) over voltage for fresh and cells retrieved from cryopreservation on day 7 through 15. (Day 7-10: Group 1, n=9 and Day 10-15: Group 2, n=6) Comparison of mean current densities of fresh chondrocytes with cells from Group 1 and Group 2 showed highest current magnitude in Group 1

(Day 7-10) and lowest current magnitude in Group 2 (Day11-15) while fresh chondrocyte mean current density fell in between them. (Figure 27)

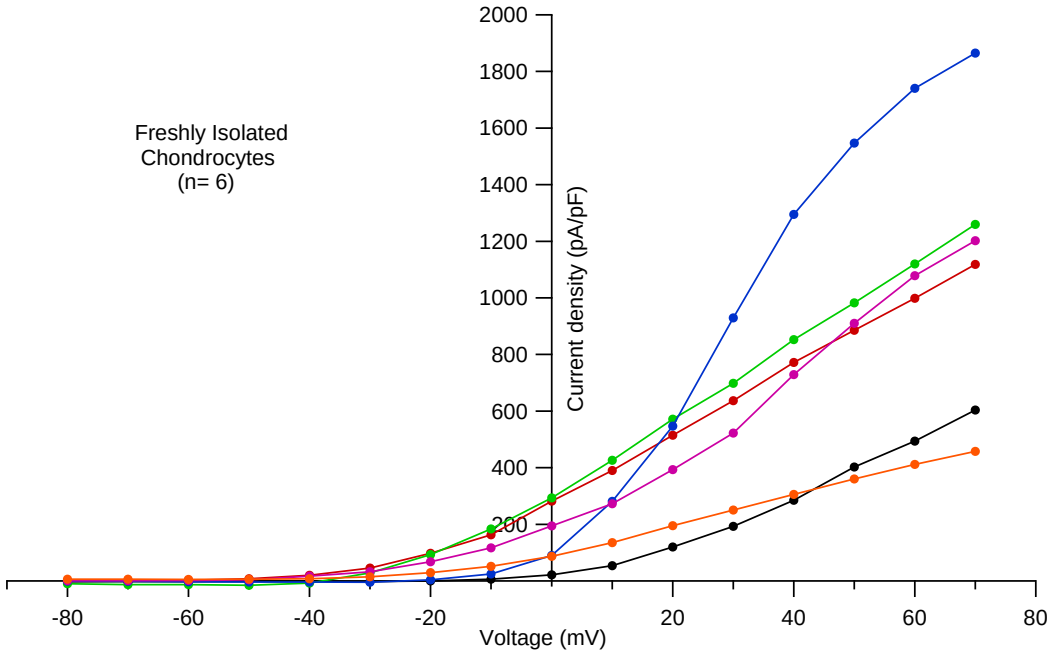


Figure 24. Displayed individual current density vs. voltage curve for fresh chondrocytes (n=6)

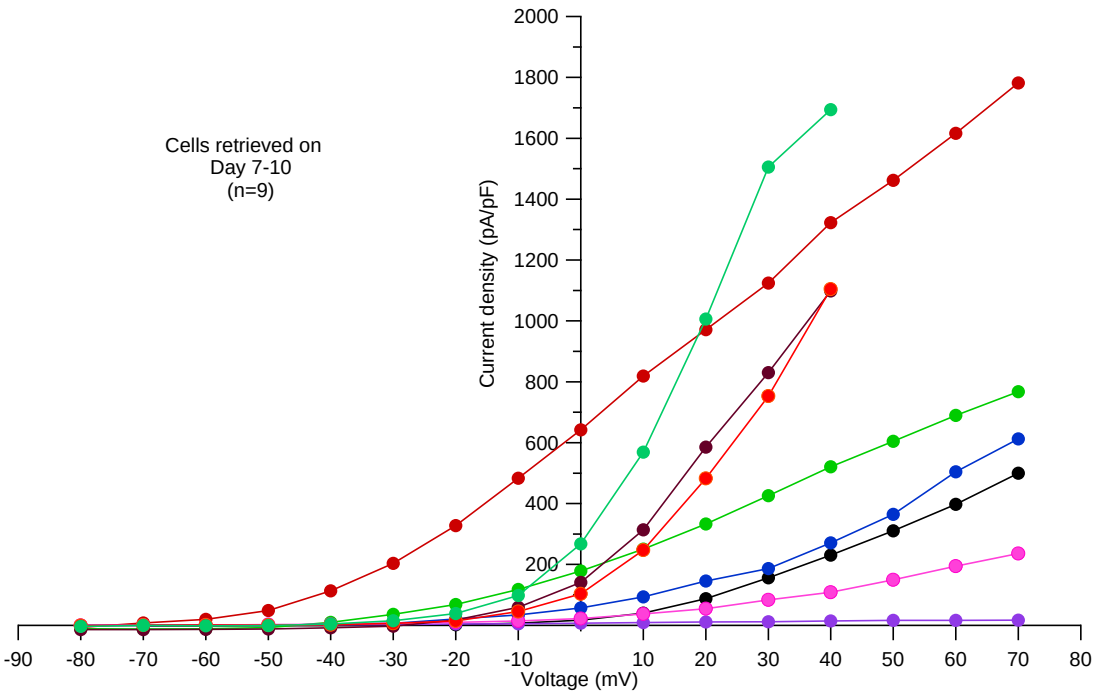


Figure 25. Displayed individual current density vs. voltage curve for chondrocytes retrieved on Day 7-10 (n=9). For n=3, the currents saturated in the last three sweeps and could not be recorded.

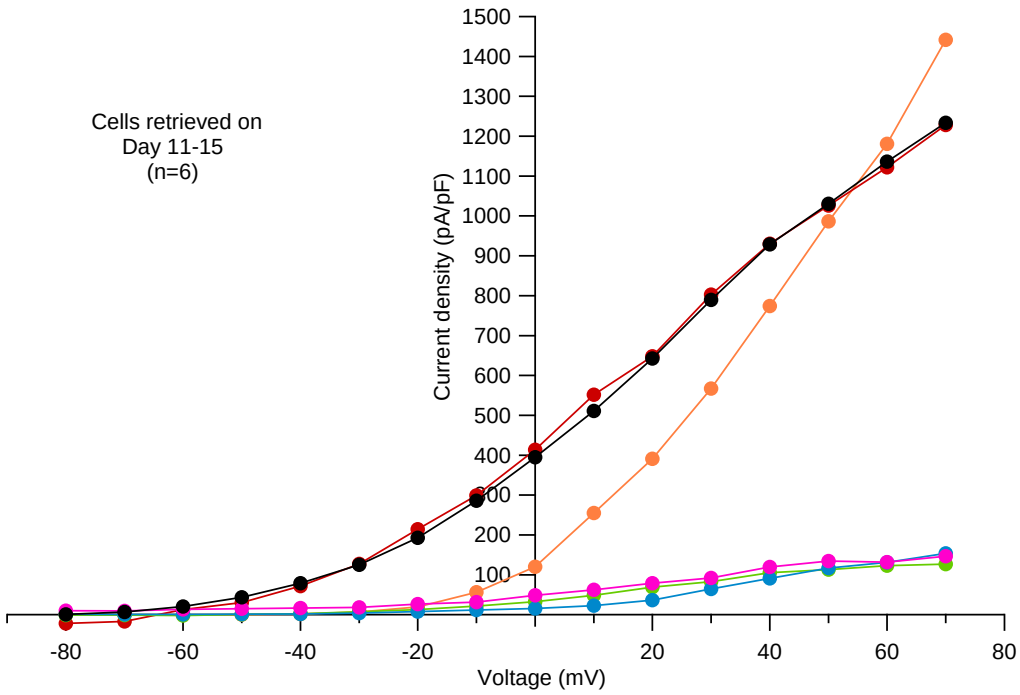


Figure 26. Displayed individual current density vs. voltage curve for chondrocytes retrieved on Day 11-15 (n=6)

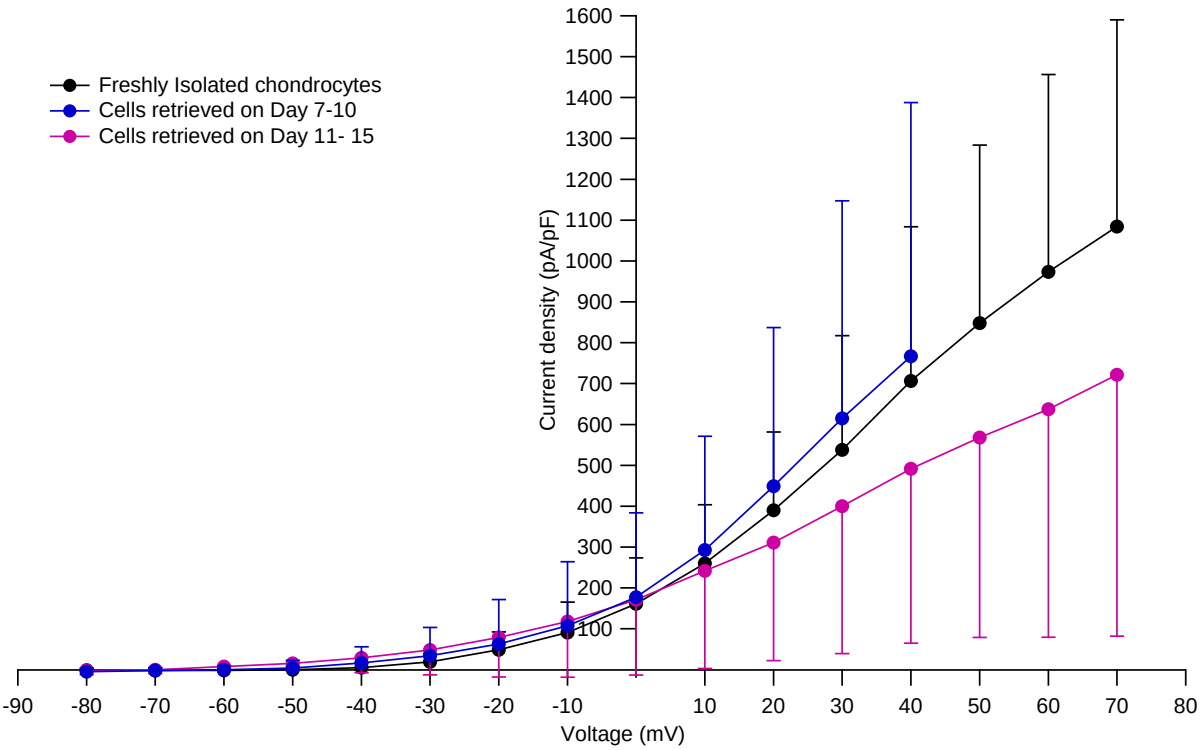


Figure 27. Displayed mean current densities vs. voltage curves for fresh and cryopreserved chondrocytes from both groups. The last 3 points for Group 1 (Day7-10) have not been plotted as the value of 'n' was unequal.

Change in current densities at significant voltages (chosen to be -10 mV, 0 mV, +10 mV, +20 mV, +30 mV, +40 mV) was found to be non significant among the three groups ie. freshly isolated cells and cryopreserved chondrocytes from Group 1 (Day 7-10) and Group 2 (Day 11-15) (Table 4)

Table 4. Displayed p values (using Kruskal- Wallis non parametric test) for change in current densities at different voltages for freshly isolated chondrocytes and croyopreserved cells from both groups. p value > 0.05 was considered significant

Voltage (mV)	P Value
-10	0.883
0	0.838
+10	0.781
+20	0.809
+30	0.661
+40	0.635

When the effect of 10mM TEA^+ was compared, it was observed that there was significant inhibition of current magnitude in freshly isolated as well as cryopreserved chondrocytes. This confirms that the currents found in both freshly isolated chondrocytes and cells retrieved from cryopreservation on various days (Group 1 and Group 2) are Voltage gated potassium currents. Figure 28 and 29 show the mean current densities with standard deviation plotted against voltage for freshly isolated chondrocytes and cryopreserved chondrocytes from Group 1. (Day 7-10) For Group 2 (Day 11-15) only one recording with

TEA^+ could be made and that has been plotted as Current density vs voltage in Figure 30

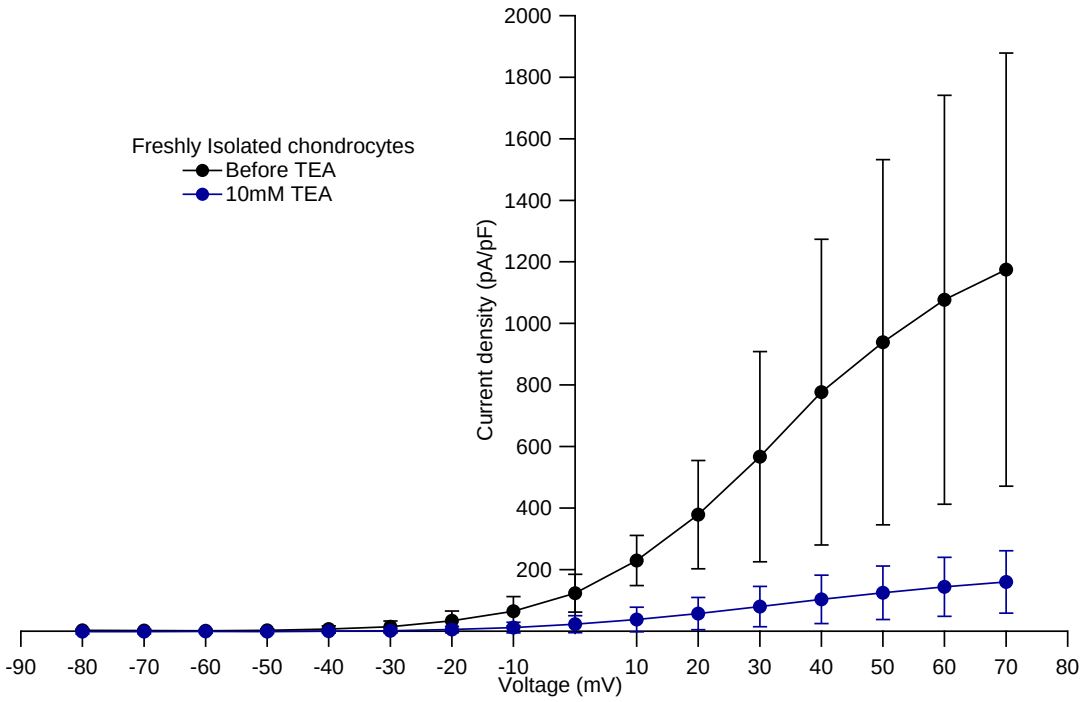


Figure 28. showing Mean current density with standard deviation vs. voltage curve before and after addition of 10mM TEA^+ in freshly isolated chondrocytes (n=3)

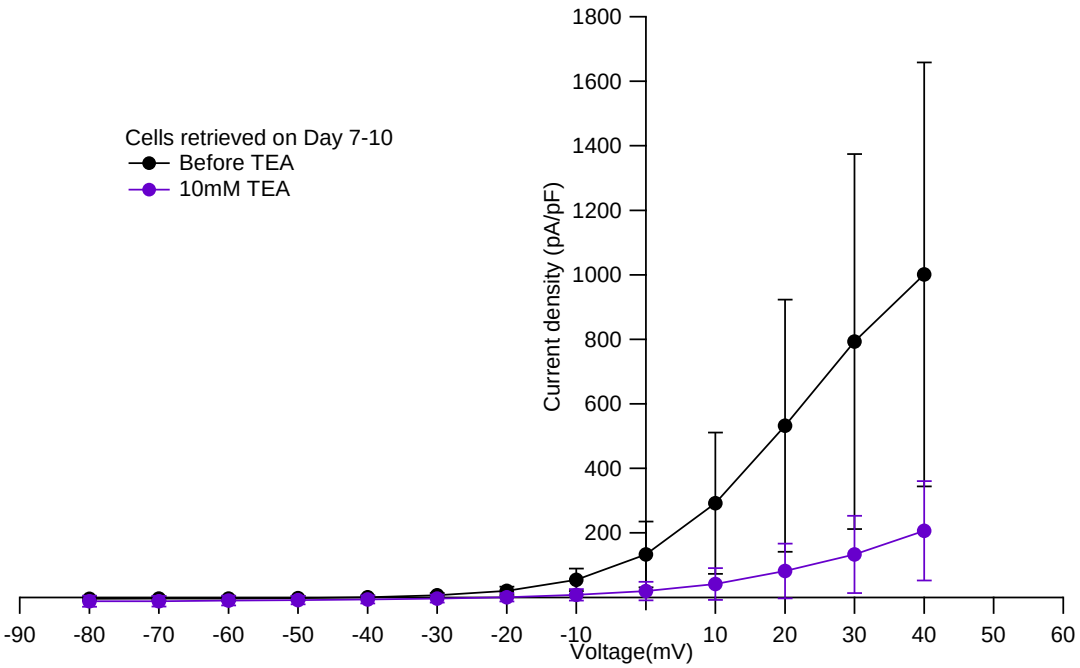


Figure 29. showing Mean current density with standard deviation vs. voltage curve before and after addition of 10mM TEA^+ in Group 1 cryopreserved cells (Day 7-10) (n=3) current density for last three seeps not plotted as values for 'n' were unequal.

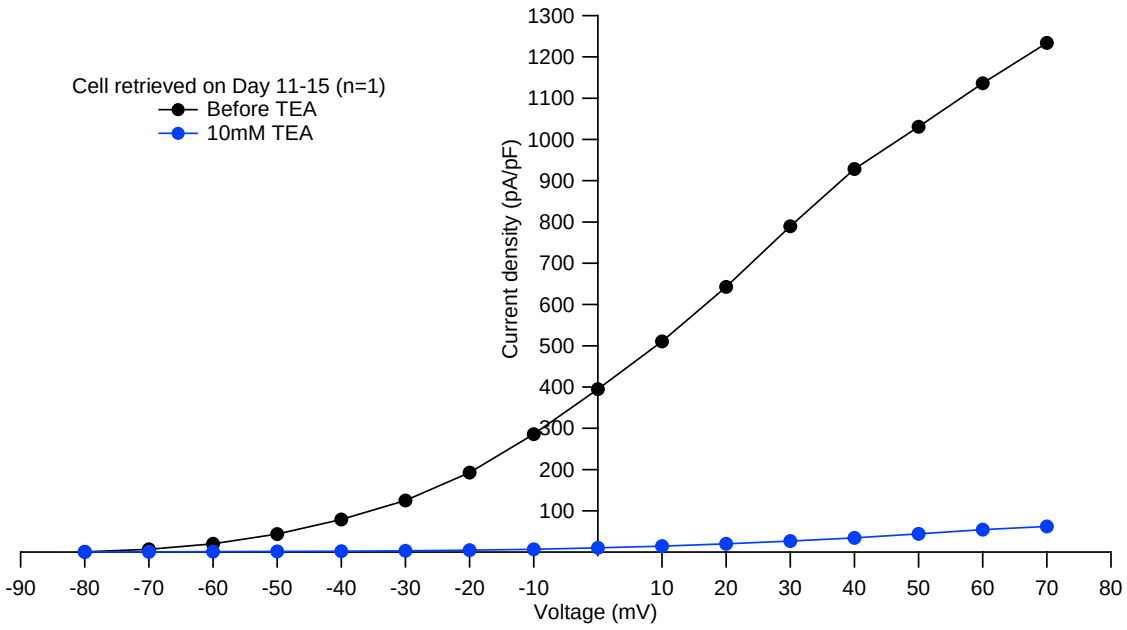


Figure 30. showing current density vs. voltage curve before and after addition of 10mM TEA^+ in a Group 2 cryopreserved cell (Day 7-10) (n=1)

When the reversal potentials were compared across various groups, it was observed that reversal potential for cryopreserved cells was in the same range as freshly isolated chondrocytes. As mentioned earlier, the measured reversal potential was near the calculated Nernst equilibrium potential for potassium. This suggests that the permeant ion responsible for the outward current seen in freshly isolated as well as cryopreserved chondrocytes is potassium. Current reversal in freshly isolated chondrocytes was observed to occur in the range of -75 mV to -69 mV (n=3) (Figure 31). For Group 1 (Day7-10) cryopreserved cells, reversal

occurred in the range of -73.5 to -62 mV (n=4) (Figure 32). For Group 2 (Day 11-15) current reversal was observed to occur at voltage ranges of -68.5 to -66.5 mV (n=2) (Figure 33)

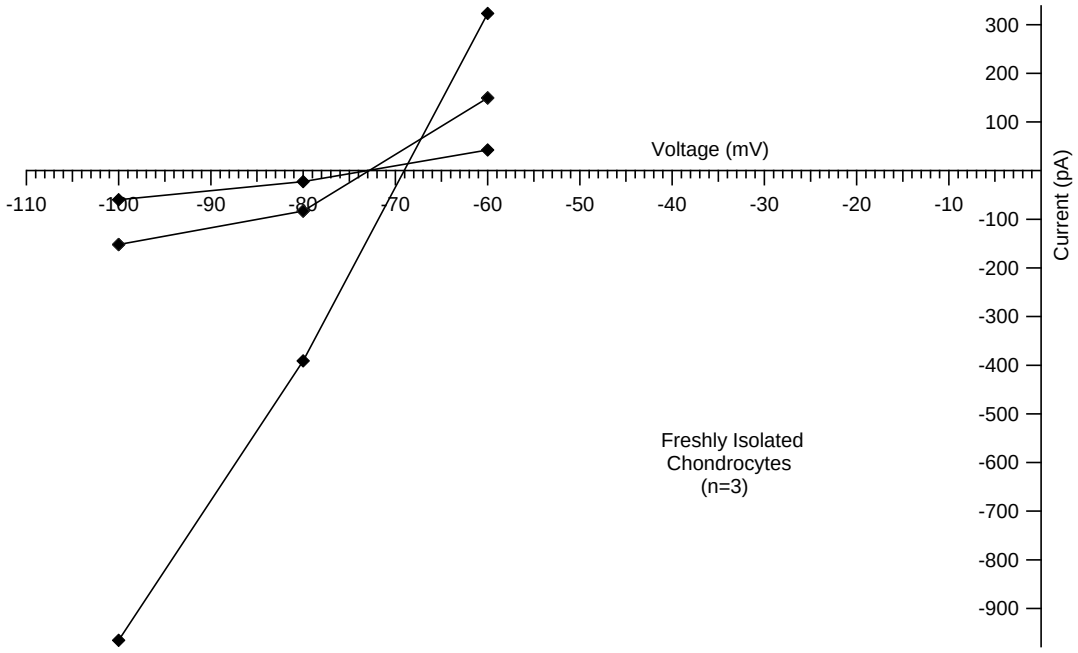


Figure 31 Displayed leak subtracted current vs. voltage graph to depict reversal potential for freshly isolated chondrocytes (n=3)

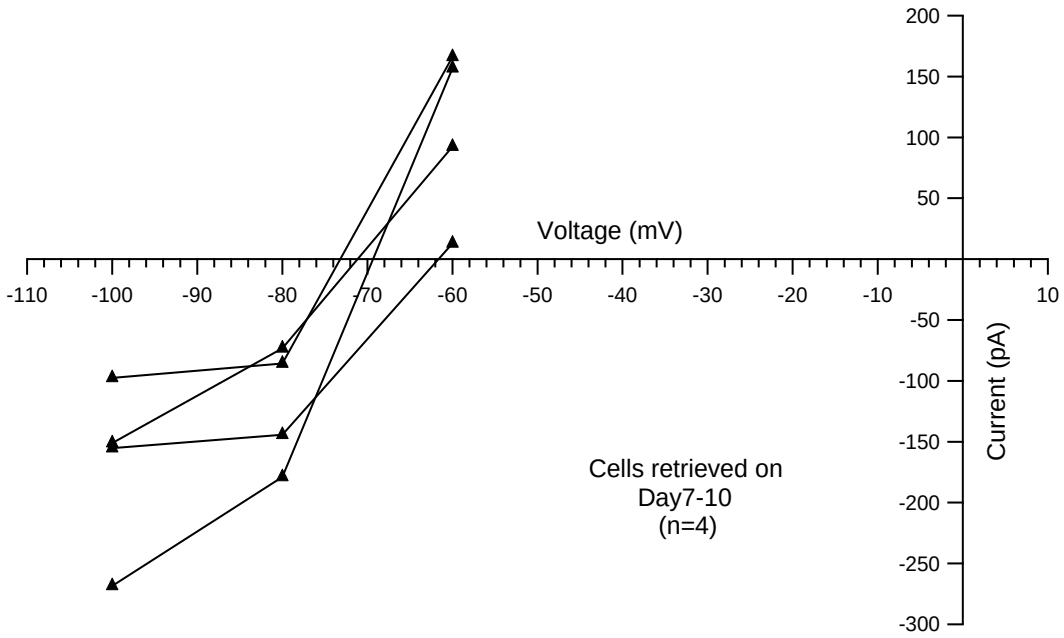


Figure 32. Displayed leak subtracted current vs. voltage graph to depict reversal potential for Group 1 cryopreserved Chondrocytes (Day7-10) (n=3)

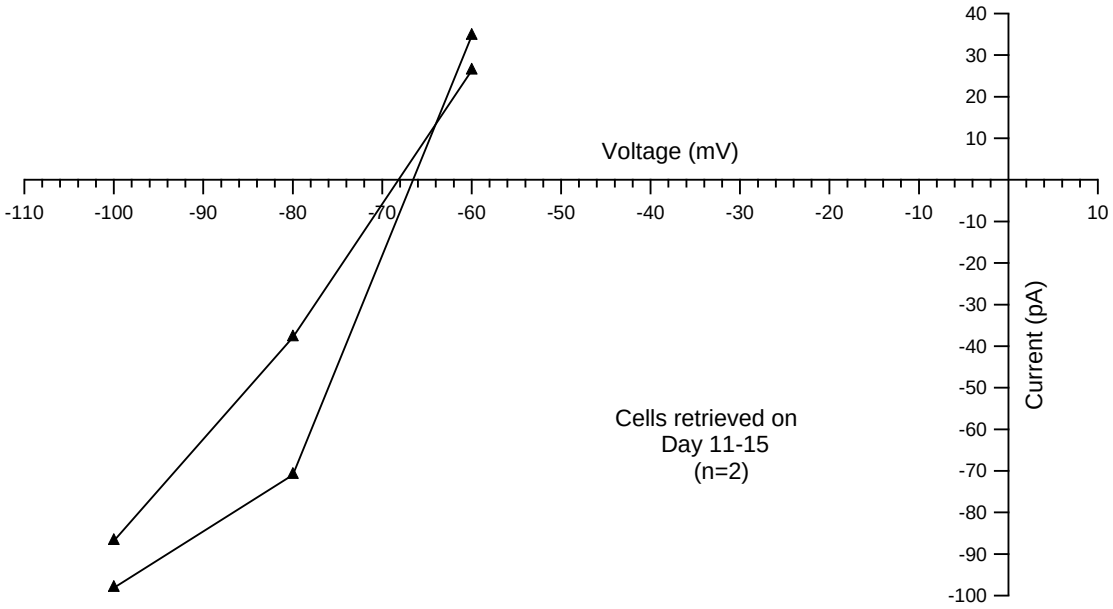


Figure 33. Displayed leak subtracted current vs. voltage graph to depict reversal potential for group 2 cryopreserved chondrocytes (Day11-15) (n=2)

Comparison of the cell capacitance measured at the beginning of each recording revealed the following results. Freshly isolated chondrocytes had a mean cell capacitance of 6.03±2.8 pF (n=6). Cryopreserved cells from Group 1 (Day7-10) showed mean cell capacitance of 6.36±2.16 pF while those from Group 2 (Day 11-15) had a capacitance of 3.61±0.83 pF (Figure 34).

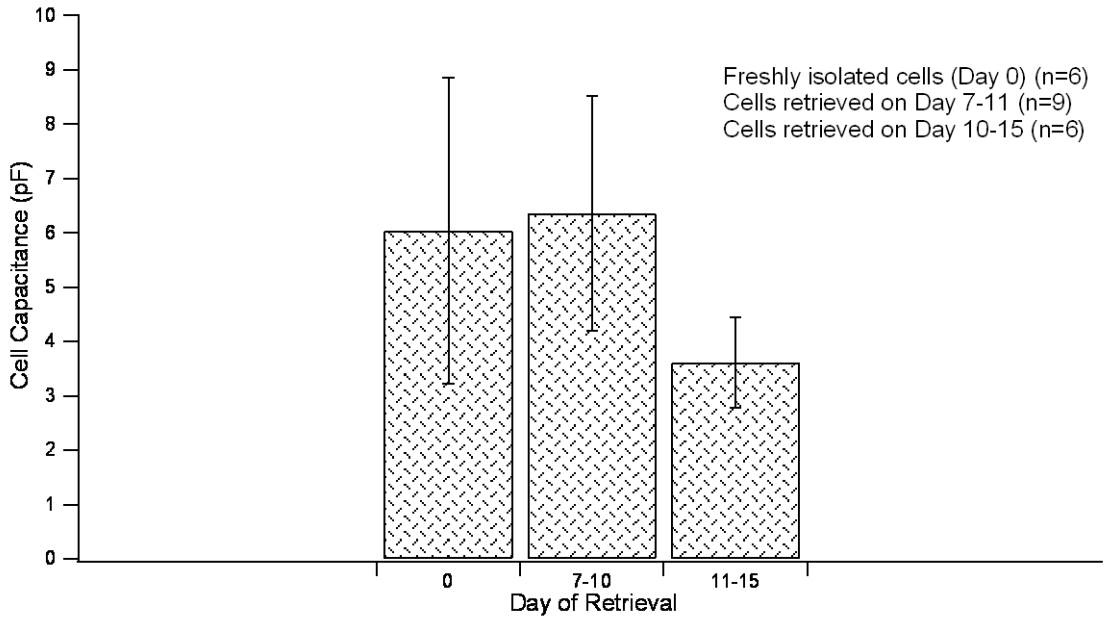


Figure 34. showing comparison of Mean cell capacitance with standard deviation of freshly isolated chondrocytes (n=6) and cryopreserved cells (Group 1-n=9, Group 2- n=6) (p value for Day 0 vs. Group 2= 0.041, and p value for Group 1 vs. Group 2= 0.003)

Results of Extended cryopreservation:

Cells retrieved from cryopreservation after 30 days showed a current profile similar to freshly isolated chondrocytes that is, presence of a voltage sensitive, slowly activating, and outwardly rectifying current. The individual current densities vs. voltage graph portraying the current profile has been shown in Figure 35.

Effect of 10 mM TEA^+ was also observed to be similar to that seen in fresh chondrocytes and cells cryopreserved for a shorter duration. The significant reduction in current magnitude over all voltages suggests presence of a channel from the voltage gated potassium sub-family. (Figure 36)

Analysis of the reversal potential showed currents reversing at voltages ranging from -77.5 to -71.5 mV which was close to calculated equilibrium potential for potassium, showing that the permeant ion species in this experiment is potassium. (Figure 37)

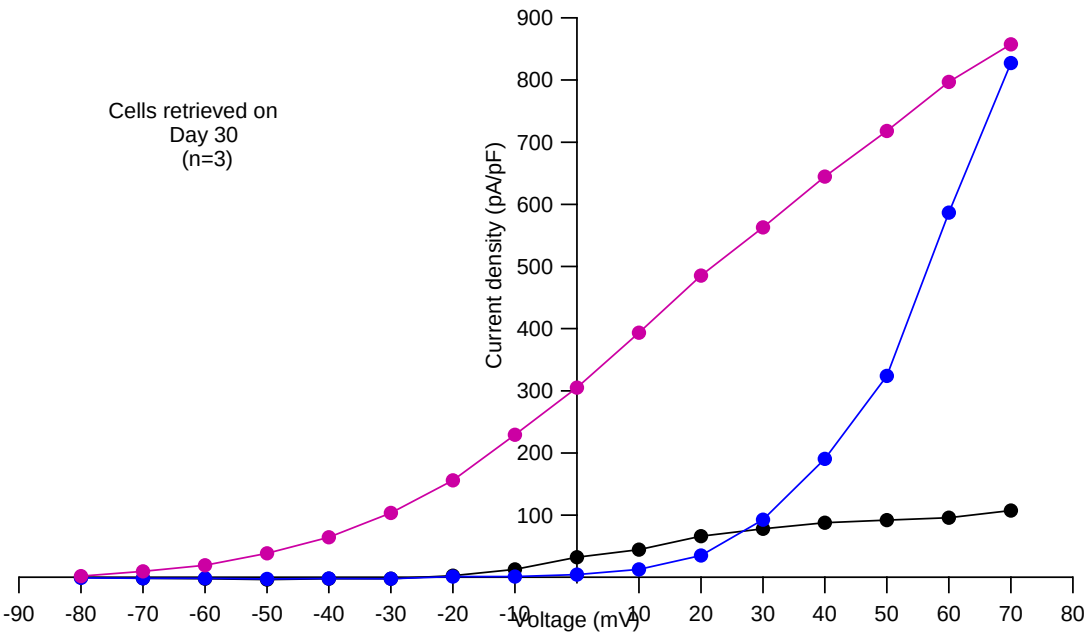


Figure 35. Displayed current density vs. voltage curves for chondrocytes cryopreserved for 30 days (n=3)

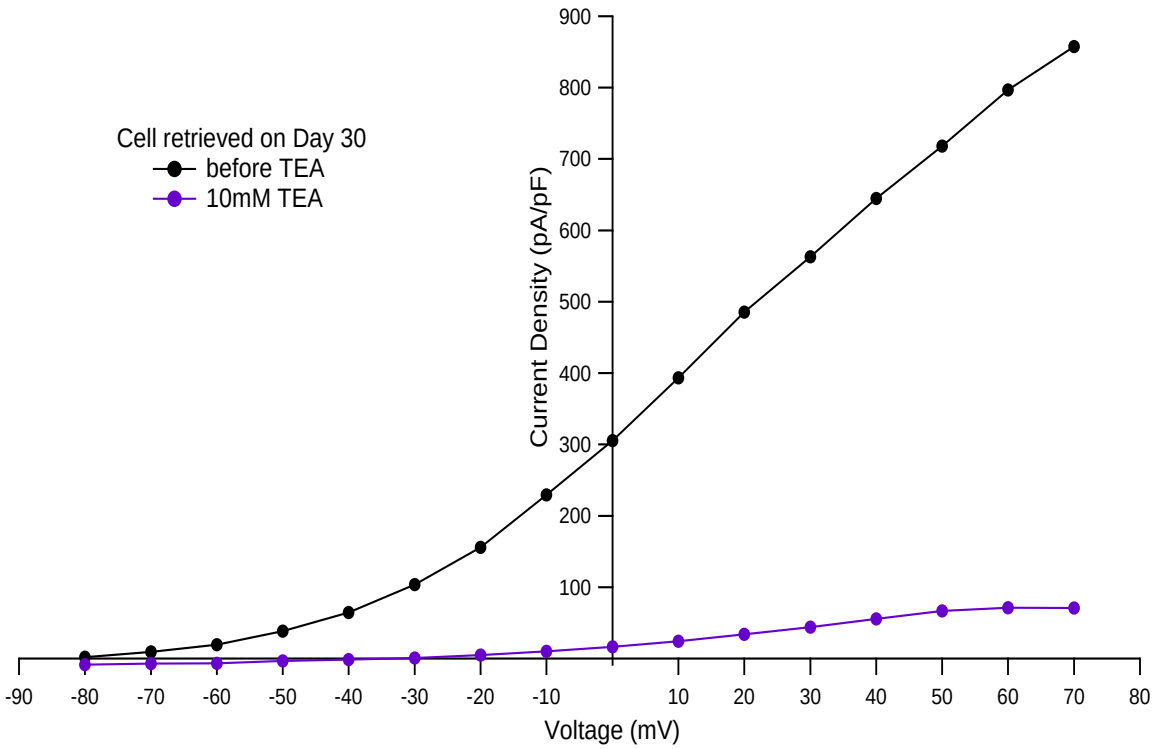


Figure 36. showing current density vs. voltage curve before and after addition of 10 mM TEA^+ in chondrocytes retrieved after 30 days of cryopreservation (n=1)

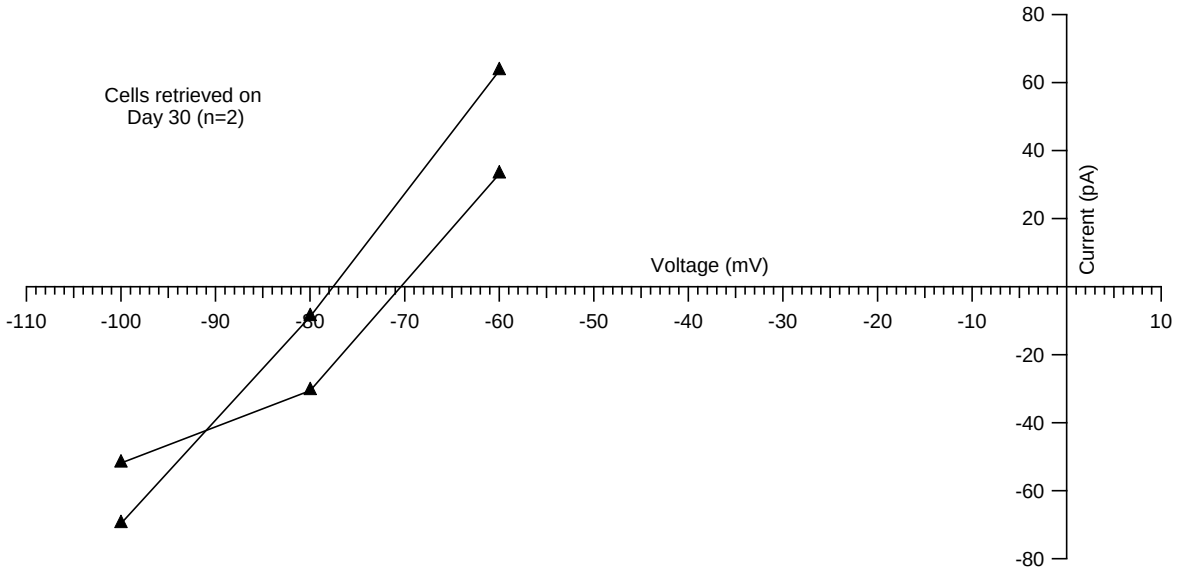


Figure 37. Displayed leak subtracted current vs. voltage graph to depict reversal potential for cryopreserved chondrocytes (Day 30) (n=2)

DISCUSSION

DISCUSSION

Subjecting freshly isolated chondrocytes to the cryopreservation protocol as explained in the methods section, yielded cells which were viable and looked morphologically similar to fresh cells. We used Dimethyl Sulfoxide (DMSO) as the cryoprotective agent which is one of the most commonly used CPAs and been used extensively for cryopreserving chondrocytes.(9,48) The concentration of DMSO used was fixed after reviewing test sample viability and also available literature.

In our experiment, 4 sets were used of which the fourth set yielded the maximum viability of 88% which dropped to 68% by Day 15. Set 1 and Set 2 showed 70% viability on Day 7 which dropped to 10% for Set1 and 66% for Set 2 by Day 15. The variation in viability can be explained in part by the condition within the liquid nitrogen container since the temperature at which individual vials remained while they were stored in the container could not be controlled. For Set 3, none of the cells could be retrieved despite using the same freezing and thawing protocol. The cause for this may be due to the cell number that was used for each aliquot. Since the cell yield from shavings was not sufficient, therefore only 0.5 million cells were used per aliquot. At this number, the concentration of DMSO used (10%) may have proved to be toxic to the cells and may have led to cell death. Also, another factor which may have led to this result is the health of the cells before they were frozen. Since the age or state of the animal (goat)

could not be confirmed, the effect of this subjective observation can only be speculated. It was observed that the number of cartilage shavings obtained from the articular surface was minimal as compared to other experiments. The number of chondrocytes isolated after digestion was also substantially less. This may indicate questionable chondrocyte health either due to age or disease. Unhealthy chondrocytes would not have tolerated the osmotic as well as the freezing stress which would have led to cell death and drop in viability.

Using a depolarizing protocol during patch clamp analysis showed the presence of a family of outward currents in both freshly isolated chondrocytes as well as cells, retrieved after cryopreservation. By convention an outward current can result either due to movement of a positive ion out of the cell or a negative ion into the cell. The solutions designed were such that the only positive ion which could give rise to such currents was Potassium and the only negative could be Chloride. The currents were originating due to the flow of potassium ions was confirmed when the use of a potassium channel blocker- TEA^{+}, caused significant reduction in current magnitude. Also, current reversal was seen to be very close to the calculated Nernst equilibrium potential for potassium which confirmed that current causing permeant ion species was potassium.

The current profile showed a slowly activating, non-inactivating and outwardly rectifying nature. Similar currents have been reported in chondrocytes in studies conducted previously. (42) Comparison of the currents seen in freshly isolated

chondrocytes and in cells retrieved from cryopreservation did not show much difference. Also the reversal potential as well as the effect of TEA^+ gave similar results as were seen with fresh cells. It can therefore be inferred that cryopreserved chondrocytes do not differ from their freshly isolated counterparts in terms of channel expression.

Cell capacitance, which has been taken as a measure of the cell size was also compared across groups. It was observed that as the cells were cryopreserved for a longer duration as in the case of Group 2 (Day 11-15) there was a reduction in the mean cell capacitance. From the results obtained from this study, it appears that the cell capacitance of freshly isolated chondrocytes and cryopreserved cells is comparable till only about 10 days of cryopreservation (p=529) following which cell capacitance shows decline (statistically significant as freshly isolated cells compared to Group 2 cell yielded a p value= 0.041 and Group 1 cells vs. Group 2 cells were seen to have a p value= 0.003 upon analysis). In order to further study this result, a larger sample size to observe the interaction of chondrocytes with the CPA used, their behavior when exposed to freezing stress, as well as a detailed electrophysiological analysis is required.

Cryopreservation for 30 days was tried for a sample from 1 set and the results obtained were similar to the ones obtained for chondrocytes cryopreserved for a shorter duration (7-15 days). The current seen was similar to the outwardly rectifying profile that is seen in fresh chondrocytes which was confirmed again to

be potassium current by the use of TEA^+ and also assessing the reversal potential. These results show that cryopreservation for short as well as relatively longer durations do not cause significant changes in channel expression. Some studies have used fresh chondrocytes and cryopreserved cells to investigate potassium channel expression by using patch clamp, PCR as well as immuno-histochemistry.(21) These studies presume that cryopreserved chondrocytes have the same functional profile as freshly isolated cells. With the result found in our study, this presumption is supported. It may be implied that chondrocytes may be preserved in this manner to bypass the issue of inadequate sample source, reduce cost of repeated isolation and maintain a chondrocyte bank for future use.

CONCLUSION

CONCLUSION

Goat articular chondrocytes can be successfully cryopreserved over short durations of upto 15 days with good retrieval and viability. Both freshly isolated as well as cryopreserved chondrocytes show the presence of currents originating from voltage gated potassium channel sub-family which show considerable inhibition with 10mM TEA^{+}. Phenotype maintenance in the form of ionic channel expression does not seem to be affected by cryopreservation in these cells as is evident by the maintenance of current profile and study of reversal potential. Chondrocytes cryopreserved over an extended period of 30 days present similar results as other cells analyzed indicating retention of functional properties but more data and further analysis will be required for making any concrete statements. In conclusion, it may be stated that cryopreserved chondrocytes can be utilized in place of freshly isolated chondrocytes when there is any limitation to their use.

LIMITATIONS

&

FUTURE

DIRECTION

LIMITATIONS & FUTURE DIRECTION

Cryopreservation protocol used in this study yielded cells with varying viabilities across sets. In future, further improvement of the protocol can ensure optimal viability in a consistent manner. It may also be extremely beneficial to further explore the effect of extended periods of cryopreservation from multiple samples as the results of cryopreservation for 30 days from one set seem to be promising. In our study patch clamp technique was used to study the Potassium channel currents in chondrocytes before and after cryopreservation. Since chondrocytes have shown the presence of numerous other channels, it would be helpful to compare whether functionality of these channels is affected by cryopreservation or not. Also, other techniques like immune-histochemistry, doubling time in culture, gene expression can be used along with patch clamp to make a decisive statement about phenotype maintenance in cryopreserved chondrocytes. The logical next step after optimization of the cryopreservation protocol would be to freeze articular cartilage shavings and analyze the functional properties of chondrocytes isolated from them. Retrieval of viable and functional chondrocytes can advance the fields of tissue engineering, reconstructive surgery and orthopedic research by leaps and bounds.

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